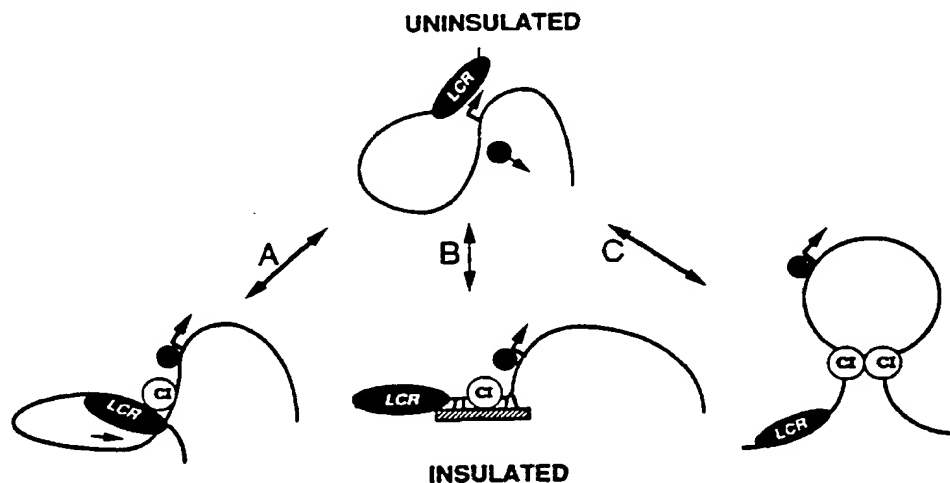


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(54) Title: DNA SEQUENCE WHICH ACTS AS A CHROMATIN INSULATOR ELEMENT TO PROTECT EXPRESSED GENES FROM CIS-ACTING REGULATORY SEQUENCES IN MAMMALIAN CELLS

**(57) Abstract**

A vector construct containing a newly-characterized insulator element isolated from the DNA of a higher eukaryotic organism is described. The insulator element of the invention comprises a DNA sequence which contains a constitutive hypervariable site whose functional activity and biochemical characterization were previously unknown. The insulator element has been demonstrated for the first time in mammalian cells to function to buffer or insulate an expressed gene from the activity of *cis*-acting regulatory elements, such as enhancers, in the surrounding DNA.

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**DNA SEQUENCE WHICH ACTS AS A CHROMATIN INSULATOR
ELEMENT TO PROTECT EXPRESSED GENES FROM *CIS*-ACTING
REGULATORY SEQUENCES IN MAMMALIAN CELLS**

5 **FIELD OF THE INVENTION**

 The present invention relates to the isolation, identification, and characterization of a DNA element residing in higher eukaryotic chromatin structural domains. The invention provides the isolation of a functional DNA sequence comprising a chromatin insulating element from a vertebrate system and provides the first employment of the insulator element as a functional insulator in mammalian cells. The invention further relates to a method for insulating the expression of a gene from the activity of *cis*-acting regulatory elements in eukaryotic chromatin.

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BACKGROUND OF THE INVENTION

 The chromosomal DNA of eukaryotic organisms is thought to be organized into a series of higher-order regions or "domains" that define discrete units of compaction of chromatin, which is the complex of nucleoproteins interacting with eukaryotic nuclear DNA. In addition to providing a means for condensing the very large chromosomes of higher eukaryotes into a small nuclear volume, the domain organization of eukaryotic chromatin may have important consequences for gene regulation. The regulation of tissue-specific gene expression at the DNA level is mediated through an interaction between regulatory sequences in the DNA of eukaryotic cells and a complex of transcriptional factors (i.e. nucleoproteins) which are specific for a particular tissue type and for a particular gene. Further, the higher-order chromatin structure of tissue-specific genes is also regulated in a tissue-specific manner (reviewed by van Holde, K.E. (1989). "Chromatin structure and transcription". In: Chromatin, K.E. van Holde, ed., New York, New York; Springer-Verlag, pp. 355-408).

 Higher-order chromatin domains may also define independent units of gene activity and regulation. For example, a discrete domain of eukaryotic

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chromatin is sometimes more than 100 kilobases in length and may encompass a particular gene or gene cluster. In those tissues where a given gene or gene cluster is active, the domain is sensitive to DNase I, thus lending support to the notion that the chromatin of an active domain is in a loose, decondensed configuration that is easily accessible to *trans*-acting factors (Lawson, G.M., Knoll, B.J., Marsh, C.J.,
5 Woo, S.L.C., Tsai, M-J. and O'Malley, B.W. (1982). "Definition of 5' and 3' structural boundaries of the chromatin domain containing the ovalbumin multigene family". J. Biol. Chem., 257:1501-1507; Groudine, M., Kohwi-Shigematsu, Gelinas, R., Stamatoyannopoulos, G. and Papayannopoulou T. (1983). "Human
10 fetal-to-adult hemoglobin switching: changes in chromatin structure of the β -globin gene locus". Proc. Natl. Acad. Sci. USA, 80:7551-7555; Jantzen, K., Fritton, H.P., and Igo-Kemenes, T. (1986). "The DNase I sensitive domain of the chicken lysozyme gene spans 24kb". Nucl. Acids Res., 14:6085-6099; and Levy-Wilson, B. and Fortier, C. (1989). "The limits of the DNase I-sensitive domain of the
15 human apolipoprotein B gene coincide with the location of chromosomal anchorage loops and define the 5' and 3' boundaries of the gene". J. Biol. Chem., 264: 21196-21204). By contrast, in those tissues where the same gene is not active, the chromatin of the domain is in a tight configuration that is inaccessible to *trans*-
20 acting factors. Thus, decondensing the higher order chromatin structure of a domain is required before regulatory factors can interact with target sequences, thereby determining the transcriptional competence of that domain.

Although very little is presently known about how the higher-order
25 chromatin structure is regulated, results from studies in physical chemistry, cell biology, and molecular biology have supported the theory that the eukaryotic genome is indeed organized into topologically isolated domains. Central to the understanding of the chromatin structure of a particular domain is how the domains
30 are precisely defined and formed. The higher order chromatin structure of genes as well as the flanking region surrounding the genes are uniform throughout each domain, but are discontinuous in the regions, loosely termed "boundaries", between adjacent domains (Eissenberg, J.C. and Elgin, S.C.R. (1991). "Boundary
35 function in the control of gene expression". TIG, 7:335-340). It is generally

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thought that domains are delimited by special nucleoprotein structures assembled at specific sites along the eukaryotic chromosome. These specific sites are believed to be the domain boundaries of chromatin.

In addition to understanding how the higher order chromatin structure of a domain is regulated as a unit, it is crucial to know how the boundaries of a domain may be organized. For example, the genome has been demonstrated to be organized into topologically isolated loops that radiate out from nuclear matrices (Benyajati, C. and Worcel, A. (1976). "Isolation, characterization and structure of the folded interphase genome of Drosophila melanogaster". Cell, 9:393-407; Paulson, J.R. and Laemmli, U.K. (1977). "The structure of histone-depleted metaphase chromosomes". Cell, 12:817-828; Gasser, S.M. and Laemmli, U.S. (1987). "A glimpse at chromosomal order". TIG, 3:16-22; and Garrard, W.T. (1990). "Chromosomal loop organization in eukaryotic genomes". In: Nucleic Acids and Molecular Biology, F. Eckstein and D.M.J. Lilley, eds. (Berlin, Springer-Verlag) pp. 163-175). It has been suggested that the higher order chromatin structure of each of these chromatin loops is independently regulated and that the ends, or boundaries, of the loops may insulate the genes in one loop from the influence of the regulatory sequences in adjacent loops. Among the many possible functions of a boundary, the most prominent function would be that of insulating genes from the *cis*-acting regulatory elements of an adjacent domain.

A. Stief et al. (1989, "A Nuclear DNA Attachment Element Mediates Elevated and Position-dependent Gene Activity", Nature, 341:343-345) have reported that an "A" element, which maps to the 5' and 3' boundaries of the region of general DNase sensitivity in the active chromatin of the chicken lysozyme gene, appeared to be a type of *cis*-acting DNA element which possessed boundary-like properties. However, the "A" element was determined to have enhancer-like activity and to activate transcription. In addition, Stief et al. used only transient transfection assays to measure chloramphenicol acetyltransferase ("CAT") activity. Further, when the "A" element was linked to a reporter gene and transfected into chicken cells in an effort to obtain stable integration, the data presented did not

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portray an authentic or correlative copy number effect, since the number of the putatively integrated plasmid DNAs was measured on an absolute scale, while relative CAT activity was measured on a logarithmic scale. In fact, there was no more actual correspondence between the copy number of the reporter gene linked to the "A" element and the amount of CAT activity observed, than there was for the reporter gene not linked to the "A" element. Consequently, the chicken "A" element was neither directly nor convincingly demonstrated to be a functional or pure insulator sequence. Further, the "A" element is a strong transcriptional activator on its own and can perturb the expression of a linked gene when integrated into host DNA.

R. Kellum and P. Schedl (1992, "A Group of scs Elements Function as Domain Boundaries in an Enhancer-Blocking Assay", Mol. Cell. Biol., 12:2424-2431) described the presence of constitutively hypersensitive sites called scs (i.e. "special chromatin structures") in the fruit fly, *Drosophila melanogaster*. The scs, considered to be putative boundary DNA segments of the 87A7 heat shock locus of *Drosophila*, were capable of blocking the action of the *D. melanogaster* yolk protein-1 enhancer when an scs was placed between it and the *hsp70* promoter. These authors showed that the scs worked to buffer the 87A7 heat shock gene from nearby regulatory sequences in transgenic *Drosophila* (Kellum, R. and Schedl, P. (1991). "A position-effect assay for boundaries of higher order chromosomal domains". Cell, 64:941-950), and that the scs by itself did not possess its own regulatory activity.

However, to date, the isolation and use of a "pure" insulator from higher eukaryotes, which, on its own, does not perturb gene expression, either positively or negatively, and which serves to insulate the expression of a given gene in a mammalian system, has not been demonstrated.

In vertebrates such as chickens, mice, and humans, the beta-globin locus has been well characterized. In all three organisms, the chromatin structure of the beta-globin locus is extremely well conserved (Fig. 1). At the very 5' end of the beta-globin locus, a constitutive DNase I-hypersensitive site (called the 5' HS5 in humans and mice, and the 5' HS4 in chickens) is present in all tissue types

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(Tuan, D., Solomon, W., Li, Q. and London, I.M. (1985). "The " β -like-globin" gene domain in human erythroid cells". Proc. Natl. Acad. Sci. USA, 82:6384-6388; Forrester, W.C., Takegawa, S., Papayannopoulou, T. Stamatoyannopoulos, G. and Groudine, M. (1987). "Evidence for a locus activation region: the formation of developmentally stable hypersensitive sites in globin expressing hybrids". Nucl. Acids Res., 15:10159-10177; and Reitman, M. and Felsenfeld, G. (1990). "Developmental regulation of topoisomerase II sites and DNase I-hypersensitive sites in the chicken β -globin locus". Mol. Cell. Biol., 10:2774-2786). The constitutive hypersensitive site is a DNA segment or region comprising a particular DNA sequence in a chromatin domain which is particularly sensitive to DNase I activity. Until the present invention, the function of the 5'-most constitutive hypersensitive site in the beta-globin locus of eukaryotic chromatin was unknown.

Further into the 5' end of the beta-globin locus, there are other types of erythroid-specific DNase I-hypersensitive sites (Tuan, D. et al., 1985, "The β -like-globin" gene domain in human erythroid cells", Proc. Natl. Acad. Sci. USA, 82:6384-6388; U.S. Patent No. 5,126,260 to I.M. London et al.; Grosveld, F. Blom van Assendelft, G., Greaves, D. and Killias, G. (1987). "Position-independent, high level expression of the human β -globin gene in transgenic mice". Cell, 51:975-985; Forrester, W.C., Takegawa, S., Papayannopoulou, T. Stamatoyannopoulos, G. and Groudine, M. (1987). "Evidence for a locus activation region: the formation of developmentally stable hypersensitive sites in globin expressing hybrids". Nucl. Acids Res., 15:10159-10177; Forrester, W.C., Novak, U., Gelinas, R. and Groudine, M. (1989). "Molecular analysis of the human β -globin locus activation region". Proc. Natl. Acad. Sci. USA, 86:5439-5443; Ryan, T.M., Behringer, R.R., Martin, N.C., Townes, T.M., Palmiter, R.D., and Brinster, R.L. (1989). "A single erythroid-specific DNase I super-hypersensitive site activates high levels of human β -globin expression in transgenic mice". Genes & Dev. 3:314-323; and Talbot, D., Collis, P., Antoniou, M., Vida., M., Grosveld, F. and Greaves, D.R. (1989). "A dominant control region from the human β -globin locus conferring integration site-independent gene

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expression". Nature, 338:352-355). In contrast to the 5'-most constitutive hypersensitive site, these additional hypersensitive sites may also be known as enhancer regions, or enhancers, or, as is particular to the erythroid lineage and the beta-globin locus, "locus control regions" ("LCRs") in higher eukaryotes, including mice, chickens, and humans. The beta-globin LCRs are required for a consistently high level of expression of the family of developmentally-regulated genes in the beta-globin locus. Studies using transgenic mice and DNA obtained from beta-thalassemia patients suggest that LCRs are required for decondensing the higher-order chromatin structure of the active beta-globin domain in erythroid tissues and for potentially activating the expression of all of the genes in the beta-globin domain. Remarkably, the influence of the LCRs allows the decondensing of chromatin over more than 200 kilobases of DNA in the 3' direction (Elder, J.T., Forrester, W.C., Thompson, C., Mager, D., Henthorn, P., Peretz, M., Papayannopoulou, T. and Groudine, M. (1990). "Translocation of an erythroid-specific hypersensitive site in deletion-type hereditary persistence of fetal hemoglobin". Mol. Cell. Biol., 10:1382-1389); yet in both chicken and human, the chromatin upstream near the 5' constitutive hypersensitive site is believed to be in a tight, condensed configuration that is inaccessible to DNase 1.

In spite of the observations and hypotheses relating to the putative activity of insulators in nonvertebrate organisms, the isolation and functional characterization of such an element or elements in higher vertebrates, including humans, need to be achieved. Until the present invention, no authentic, pure, and functional vertebrate chromatin insulator element has been isolated or demonstrated to operate successfully as an insulator in a mammalian system. Furthermore, until the present invention, no clear, direct insulator function has been specifically ascribed to a vertebrate constitutive hypersensitive site, nor has such a pure insulator element been isolated, characterized, and functionally employed in mammalian cells.

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SUMMARY OF THE INVENTION

The present invention provides the first characterization and isolation of a 5' constitutive hypersensitive site of the chicken beta-globin domain and demonstrates that this region, and portions thereof, comprise a pure insulator element which forms a part of the 5' boundary of the beta-globin domain and which, on its own, does not influence gene expression in a positive or negative manner. The insulator element of the invention prevents or blocks the spread of the LCRs' disruption of chromatin in the 5' direction. Given the similarities and conserved nature of the sequences of higher vertebrates in the beta-globin locus and in regions 5' of this locus, the insulator elements of the present invention may be able to be isolated from different cell types in a variety of species.

The present invention pertains to the function and mechanism of insulation of the expression of a given gene by an isolated DNA insulator element in higher eukaryotic organisms, including humans. The present invention has achieved the isolation and use of the first vertebrate DNA element and a core DNA sequence therein having demonstrably pure insulator activity in human cells, while not perturbing the expression of a linked gene. The insulator element of the invention corresponds to a constitutive hypersensitive site at the very 5' end of chromatin in chickens, and is located about 12 kb upstream of the chicken rho-globin gene and about 18 kb upstream of the chicken beta-globin gene. In humans, a similar insulator sequence is located about 20 kb upstream of the epsilon-globin gene and about 60 kb upstream of the beta-globin gene (see Fig. 1). The nucleotide sequence of a core DNA segment of the insulator element (Seq ID No:1) of the invention is represented in Fig. 2. This core DNA sequence has insulator activity. Preferred insulator elements of this invention comprise DNA segments or elements substantially homologous to at least a portion of this sequence.

It is an object of the present invention to provide a method for using the newly-characterized 5' constitutive hypersensitive site (also termed "insulator element" or "insulator sequence" or "insulator" herein) of higher eukaryotic DNA to insulate or buffer the expression of a reporter gene from the action of an LCR. In fact, insulation using the higher eukaryotic insulator element in human cells is

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shown by the present invention to be accomplished by preventing the formation of a hypersensitive site at or near the promoter of the reporter gene. As the first pure insulator to be demonstrated to function in human cells, the present insulator element promises to be a useful tool in gene therapy and gene transfer techniques, as well as in gene regulation studies.

5 It is another object of the present invention to provide genetic expression constructs or vectors which are designed to contain one or more operational DNA sequence insulator elements comprising a DNA constitutive hypersensitive site which can insulate or buffer the activity of a particular gene
10 from the effects of the activity of *cis*-acting regulatory elements, such as enhancer or silencer regions of the DNA. The constructs may contain one or more insulator elements and one or more reporter genes in the form of transcription units, including at a minimum, an enhancer, a promoter, and a reporter gene. The
15 insulator element-containing constructs allow for the transfection of cells of a particular lineage or of a particular tissue type, depending upon the gene to be transfected and upon other features of the construct which may be cell- or tissue-specific, such as specific promoter or enhancer elements, or upon particular
20 regulatory molecules, proteins, or factors which are produced by a particular cell or tissue type and which influence the expression of a given transfected gene. In accordance with the invention, the insulator elements, reporter gene(s), and transcription unit may be provided in the form of a cassette designed to be
25 conveniently ligated into a suitable plasmid or vector, which plasmid or vector is then used to transfect cells or tissues, and the like, for both *in vitro* and *in vivo* use.

It is a further object of the present invention to provide a mechanism and a tool to restrict the action of *cis*-acting regulatory elements on genes whose
30 activities or encoded products are needed or desired to be expressed in certain cells and tissues. The genes to be insulated and expressed may be transfected into cells by using the constructs or vectors achieved by the present invention in which one or more insulator elements in a chromatin domain are strategically positioned so as
35 to buffer the transfected genes from the influence of the action of other DNA

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sequences from different chromatin domains located in *cis*.

Another object of the present invention is to provide the first pure insulator element to function solely as an insulator element in human cells. Accordingly, the invention has important practical implications for human gene therapies.

5 It is a further object of the invention to provide a method and constructs to insulate the expression of one or more transfected genes at the same or at different levels, preferably two genes encoding different proteins or protein subunits, wherein the equivalent expression of the genes is regulated by one or
10 more enhancer elements, or wherein the product of at least one gene in the construct is expressed at high levels and the product of the other gene is expressed at lower levels in the cells into whose genomes the transfected construct has integrated.

15 The present invention also provides a means for transfecting a number of different constructs, each carrying different insulator elements, transcription units, and reporter genes into a cell or tissue.

Also provided is a kit or kits containing the vector constructs of the invention and used to insulate the expression of a transfected gene or genes
20 integrated into host DNA.

The invention further provides a method and constructs to insulate the expression of a gene or genes in transgenic animals such that the transfected genes will be able to be protected and stably expressed in the tissues of the
25 transgenic animal or its offspring, for example, even if the DNA of the construct integrates into areas of silent or active chromatin in the genomic DNA of the host animal.

30 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Maps and comparison of the human and chicken beta-globin domains showing the well-conserved chromatin structure between the two species. The location of the 5' constitutive hypersensitive sites (i.e. human 5'HS5 and chicken
35 5'HS4) are depicted by black arrows. The erythroid-specific enhancer regions or

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LCRs are depicted by gray, stippled arrows. The location of the human LCR is delineated.

Figure 2: Nucleotide sequence of the 242 base pair (bp) chicken chromatin insulator element or 5' constitutive hypersensitive site.

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Figure 3A and 3B: The chicken constitutive hypersensitive site insulates the γ -neomycin (G418) resistance gene from the action of the LCR. Vector constructs were designed to contain zero, one or two copies of the 5' constitutive hypersensitive site, represented by the circle labeled "C"; an LCR element, represented by the oval labeled "LCR", and the gamma globin promoter operably associated with the neomycin gene (i.e. "the gamma-neo gene segment"), represented by the rectangle labeled " γ -NEO".

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(3A) The human erythroleukemic cell line K562 was stably transfected with the constructs shown on the left and grown in semisolid agar medium supplemented to contain G418. G418 resistant colonies were counted after 2 to 3 weeks. The relative numbers of G418 resistant colonies resulting from transfection with each construct are shown on the right. The number of colonies resulting from transfection with pJC3-4, which contains no constitutive hypersensitive site, was arbitrarily set to 1.0. The 1.2 kb fragment containing the chicken constitutive hypersensitive site is marked "C." The control fragment, a 2.3 kb *HindIII-HindIII* fragment from λ phage DNA, is marked " λ ." The arrow at the 5' end of the gene labelled " γ -NEO" indicates γ -globin promoter of the G418 resistance reporter gene. LCR indicates the location of the mouse 5'HS2.

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(3B) The constructs shown on the left include the hygromycin internal control and were transfected into K562 cells. Cells were grown in semisolid agar medium supplemented to contain either hygromycin or G418. The ratio of G418 to hygromycin-resistant colonies for each construct is shown on the right ("Neo/Hyg colony ratio"). The arrow at the 5' end of the gene labeled "TK-HYG" indicates the HSV thymidine kinase promoter of the hygromycin resistance reporter gene.

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Figure 4: Insulator function and proper positioning of the chicken 5' constitutive hypersensitive site in the constructs transfected into a human erythroleukemia cell line K562. The chicken constitutive hypersensitive site insulates the γ -globin promoter at the RNA level. Clonal cell lines containing the constructs 3-4Neo/Hyg (e.g. clones 1 and 2) and 5-4Neo/Hyg (e.g. clones 3, 4, and 5) from the experiment described in Figure 3B were established by selection in hygromycin-containing agar medium. The levels of neomycin resistance gene RNA and hygromycin resistance gene RNA were analyzed by RNase protection assays.

Figure 5A and 5B: The chicken and human 5' constitutive hypersensitive sites insulate in a directional manner.

(5A) The constructs shown on the left were stably transfected into K562 cells and G418 resistant colonies were counted as described in Figure 3A and 3B. The relative number of G418 resistant colonies is shown. The number of colonies from pJC17 was arbitrarily set to 1.0.

(5B) The constructs shown on the left were stably transfected and analyzed as in (5A). The relative number of G418 resistant colonies is shown. The number of colonies from pJC20 was arbitrarily set to 1.0.

Figure 6A and 6B: The mechanism of insulation involves the ability of the insulator element to interfere with the LCR's disruption of the nucleosome in the promoter region of the DNA. Accessibility of the restriction endonuclease *ApaI* to cut at its restriction site in the gamma globin promoter was assessed. Bands created by the cutting of *ApaI* at endogenous gamma A, gamma G, gamma-neo promoter sites are indicated with an asterisk.

(6A) Nuclei of pooled clones (about 100 to 200 clones) from the transfection of cells as described in Figure 3B were either mock-digested (lanes 1, 4, and 7) or digested with *ApaI* (lanes 2, 5, and 8) before genomic DNA was isolated. The bands generated by *ApaI* cutting in nuclei are marked γ^G for the endogenous γ and γ^A globin promoters and γ -Neo* for the γ -neomycin promoter (see Figure 6B legend for details). In lanes 3, 6, and 9, the genomic DNA was

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isolated prior to being digested to completion with *Apa*I. The percentage of cutting at each *Apa* I site as determined by a phosphoimager from the Southern blot in (A) is shown on the right for each construct. The percentage was determined by dividing the intensity of the bands in lanes 2, 5, and 8 by that of the corresponding bands in lanes 3, 6, and 9. The genomic DNA for all 9 lanes was digested with

5 *Bgl*III and *Xba*I to generate the parental bands.

(6B) Maps of the transfected DNA as well as the endogenous γ -globin genes are shown. The squares flanking the γ -neomycin gene indicate the location of either the λ control DNA (in 3-4Neo/Hyg) or the chromatin insulator (in 13-1Neo/Hyg and 5-4Neo/Hyg). The TK-hygromycin gene which is linked to

10 the γ -neomycin gene is not shown here (see Figure 3B). The probe, which is derived from the γ -globin promoter, is shown. In addition, the expected fragments observed in the Southern blot in (6A) are designated beneath each map.

15 **Figure 7:** Three models depicting possible mechanisms of insulation.

DESCRIPTION OF THE INVENTION

One aspect of the invention provides the first isolation,

20 characterization, and use of a 5' chromatin insulator element situated at the 5' boundary of the chicken beta globin locus or domain. The insulator element of the invention comprises a constitutive hypersensitive site of the DNA (also called a constitutive hypersensitive region or constitutive hypersensitive DNA segment) that

25 is capable of directionally insulating a reporter gene (i.e. a gene of interest) from a nearby regulatory element in chromatin, such as an enhancer or silencer. Although the general position of the region designated 5'HS4 was determined to be upstream and far 5' of the chicken beta-globin site at -21.5 kb (D. Tuan et al., 1985, Proc.

30 Natl. Acad. Sci. USA, 82:6384-6388), its isolation, function, and activity as an insulator element remained unknown and undetermined until the present invention.

In accordance with the invention, the sequence of the chicken constitutive hypersensitive site (i.e. insulator element) is GC-rich, unlike the "A" element of chicken and the scs of *Drosophila melanogaster*, which are AT-rich,

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thus indicating that the insulator element of the invention is a distinct and different element.

The insulator element is a control element which insulates the transcription of genes placed within its range of action. The particular pure insulator element of the invention is a DNA segment which encompasses a 1.2 kb
5 fragment of DNA isolated from the far 5' end of the chicken beta-globin locus and corresponds to the chicken 5' constitutive hypersensitive site (5'HS4). The insulator element comprises a "core" DNA sequence of about 242 bp (Seq ID No: 1), also having demonstrable pure insulator activity. The 5'HS4 site is located
10 about 12 to about 15 kb 5' of the rho-globin gene and about 18 to about 20 kb 5' of the chicken beta-globin gene.

In its natural position, the chromatin insulator element presumably buffers the genes and the regulatory machinery of one domain from the *cis*-acting
15 influence(s) of the chromatin structure and the regulatory machinery of an adjacent domain. In the genetic constructs of the invention, the insulator element can exert its optimal insulation or buffering effects on a reporter gene when the element or a DNA fragment containing the element (i.e., the 1.2 kb *SacI*-*SspI* DNA fragment isolated from a plasmid pCBGC (Reitman, M. and Felsenfeld, G. (1990), Mol.
20 Cell. Biol., 10:2774-2786) is inserted on either side of a reporter gene, such that the insulator is positioned at least about 200 bp to about 1 kb, preferably about 330 bp, from the promoter and at least about 1 kb to about 5 kb, preferably about 2.7 kb, from the promoter, at the 3' end of the reporter gene. Those skilled in the art
25 would be aware that the distances of the insulator element from the promoter and the reporter gene in the constructs are provided for guidance and may depend upon the relative sizes of the reporter gene or genes, the promoter, and the enhancer, or LCR, used in the constructs.

The isolated DNA sequence (Seq ID No:1) comprising at least a
30 portion of the isolated insulator element in chickens is shown in Fig. 2. This core DNA sequence, which provides high levels of *cis*-acting insulator activity, comprises a DNA segment comprising 242 bp. Deletion of the 242 bp sequence results in a substantial loss of insulator activity, thus indicating its role in the
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insulator function of the invention. The 242 bp sequence is extremely GC-rich and, throughout its length, contains a number of repeating motifs to which one or many nucleoproteins may bind. Preferred insulator sequences comprise DNA sequences substantially homologous to at least a portion of this sequence. Smaller portions of the 242 bp insulator sequence may also possess insulator function. Because of the repeating sequence motifs interspersed throughout the insulator element, it is likely that a number of smaller portions or fragments of the sequence containing these motifs may be used and still retain nearly complete insulator function. Further, the insulator element may be modified by base mutations including deletions, additions, or substitutions that do not substantially affect its insulating activity. Accordingly, the insulator element of this invention comprises any active DNA sequences having substantial homology to all or a part of the region of the chromatin containing the isolated 242 bp sequence of the insulator region and to the isolated 1.2 kb *SacI-SspI* insulator fragment containing the chicken 5'HS4 constitutive hypersensitive site.

The insulator elements can be employed to provide novel constructs for efficient isolation and protection of genes and for the production of a particular protein or other molecule encoded by a gene used in the constructs in cells. The insulator element of the invention may also be used to insulate particular genes introduced and subsequently expressed in transgenic animals. Constructs containing the insulator elements of the invention may be introduced into early fetal or embryonic cells for the production of transgenic animals containing the functional insulator element and reporter gene transcription unit. By insulating a gene or genes introduced into the transgenic animal, the expression of the gene(s) will be protected from negative or inappropriately positive regulatory influences in the chromatin at or near the site of integration.

In general, the constructs of the present invention contain a higher eukaryotic insulator element, an enhancer element or LCR, and a transcription unit comprising, at a minimum, a gene of interest, for example, a gene encoding a protein or precursor thereof, and a promoter to drive the transcription of the gene of interest, and other sequences necessary or required for proper gene transcription

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and regulation (e.g. start and stop sites, splice sites, polyadenylation signal, and an origin of replication). The enhancer element or LCR is located in sufficient proximity to the transcription unit to enhance the transcription thereof. The constructs may contain more than one insulator element, preferably in tandem, which are positioned so as to insulate the reporter gene and its transcription unit from surrounding DNA at the site of integration.

Transcriptionally competent transcription units can be made by conventional techniques. In general, the insulator element is placed in sufficient proximity to the enhancer or LCR so that it is functionally active to buffer the effects of a *cis*-acting DNA region on the promoter of the transcription unit. In some cases, the insulator can be placed distantly from the transcription unit. In addition, the optimal location of the insulator element can be determined by routine experimentation for any particular DNA construct. The function of the insulator element is substantially independent of its orientation, and thus the insulator can function when placed in genomic or reverse genomic orientation with respect to the transcription unit, as long as the insulator is placed preferably on both sides of a gene so as to insulate the gene from the effects of *cis*-acting DNA sequences of chromatin.

In one embodiment, in which the insulator element may be used to insulate the expression of a reporter gene, the insulator element is placed 5' or upstream of the enhancer or LCR and the promoter; a second insulator element may also be placed 3' or downstream of the reporter gene segment, as exemplified by the following linear diagram and as also exemplified by plasmid pJC19 of Fig. 5. (I E $\overrightarrow{\text{P}}$ G (I), where I = Insulator element; E = Enhancer element or LCR; $\overrightarrow{\text{P}}$ = Promoter and direction of transcription; G = gene of interest; and (I) = insulator element at end of gene). More than one insulator element may be used, preferably in tandem, if necessary or desired (see Fig. 3A and 3B).

In another embodiment, the constructs may contain more than one reporter gene whose expression is to be insulated by the insulator elements. In the case where two genes are to be transcribed and expressed at different levels, the construct may contain different enhancers to regulate the transcription of each

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gene. Accordingly, one enhancer could be a weak enhancer and the other enhancer could be a strong enhancer to allow the differential expression of the two genes in the same construct following integration into the DNA. Alternatively, the promoter of one gene can be inducible, while the promoter of a second gene can be non-inducible, or the second promoter can also be inducible, but can be induced by a different agent. Thus, as an example, the insulator is preferably placed between the weak enhancer (E_2) for the gene to be transcribed in the 3' to 5' direction (G_2) at lower levels, and the strong enhancer (E_1) for the gene to be transcribed in the 5' to 3' direction (G_1) at higher levels, as these sequences are positioned in the construct and exemplified in the following linear diagram: (I) G_2 \nwarrow E_2 I E_1 \nearrow G_1 (I). The symbol (\nwarrow) indicates the promoter and direction of transcription in the 3' to 5' direction. Further, a second insulator element may be placed near each gene at either end of the construct, as depicted by (I) in the diagrams above. In addition, the construct may contain more than one insulator element, preferably in tandem, in those parts of the construct in which an insulator is placed. As a variation of the construct which allows for different levels of expression of two different genes contained in the same construct, the insulator may be placed 5' of the promoter for the gene to be transcribed in one direction (e.g. 5' to 3'), and 3' of the enhancer for the gene to be transcribed in the opposite direction (e.g. 3' to 5'). In this variation, the enhancer may regulate both genes, as exemplified in the following linear diagram: (I) G_2 \nwarrow E I \nearrow G_1 (I). Similarly, the construct may contain more than one insulator element, preferably in tandem, in the regions in which an insulator is positioned.

In yet another embodiment, the insulator element of the invention may be used to produce constructs in which two different genes or gene subunits are transcribed and expressed at the same levels. In such constructs, a common enhancer would be positioned between the promoters for each gene and would regulate the expression of each gene, and one or more insulator elements would be placed at the ends of the genes as depicted in the following exemplary diagram: (I) G_2 \nwarrow E \nearrow G_1 I). The constructs as described herein may be used in gene transfer and gene therapy methods to allow the protected expression of a given

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gene that is stably transfected into the cellular DNA. The constructs of the invention would not only insulate a transfected gene or genes from the influences of DNA surrounding the site of integration, but would also prevent the integrated constructs from impacting on the DNA at the site of integration and would therefore prevent activation of the transcription of genes that are harmful or detrimental to the cell.

The specificity of the constructs of the invention involves transfecting the particular gene(s) of interest into a cell type having the appropriate milieu for transcription of the gene(s) whose products are desired to be expressed. The constructs of the invention are capable of being transfected into a variety of cell and tissue types. In addition, since the insulator element itself is not cell or tissue specific, it is a universal element which can act as a part of the constructs of the invention to insulate gene expression in the absence of strict cell or tissue specificity. The constructs can be designed to contain the appropriate regulatory sequences and all of the necessary DNA elements for expression of a gene of interest in a given cell type.

For assembly of the construct, the insulator element for ligation can be positioned in accordance with the desired use of the constructs of the invention. Thus, as disclosed above, at least one insulator may be positioned between an enhancer element or LCR and a transcription unit, or the insulator can be otherwise positioned on either side of a gene so as to obtain optimal insulation of the gene or genes desired to be transcribed. The insulator element can be obtained from natural sources or by synthetic means. For example, the insulator element can be excised from genomic DNA clones of eukaryotes, including chickens, mice, and humans, and the like, and then ligated with segments of DNA comprising the enhancer or LCR and the transcription unit. Alternatively, the insulator element can be synthesized according to all or part of the sequence provided in Fig. 2 by conventional techniques of DNA synthesis such as the phosphite triester chemistry method (for example, see U.S. Patent No. 4,415,732 to Caruthers et al.; and Sinha, N.D. et al., 1984, Nucl. Acids Res., 12:4539-4557).

Those skilled in the art will appreciate that a variety of enhancers,

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promoters, and genes are suitable for use in the constructs of the invention, and that the constructs will contain the necessary start, termination, and control sequences for proper transcription and processing of the gene of interest when the construct is introduced into a mammalian or a higher eukaryotic cell. The constructs may be introduced into cells by a variety of gene transfer methods
5 known to those skilled in the art, for example, gene transfection, microinjection, electroporation, and infection. In addition, it is envisioned that the invention can encompass all or a portion of a viral sequence-containing vector, such as those described in U.S. Patent No. 5,112,767 to P. Roy-Burman and D.A. Spodick, for
10 targeted delivery of genes to specific tissues. It is preferred that the constructs of the invention integrate stably into the genome of specific and targeted cell types.

Further, the DNA construct comprising the insulator element, enhancer or LCR, and transcription unit may be inserted into or assembled within a
15 vector such as a plasmid or virus, as mentioned above. The construct can be assembled or spliced into any suitable vector or cosmid for incorporation into the host cell of interest. The vectors may contain a bacterial origin of replication so that they can be amplified in a bacterial host. The vectors may also contain, in addition to a selectable marker for selection of transfected cells, as in the
20 exemplary constructs, another expressible and selectable gene of interest.

Vectors can be constructed which have the insulator element in appropriate relation to an insertion region for receiving DNA encoding a protein or precursor thereof. The insertion region can contain at least one restriction enzyme
25 recognition site.

A particularly useful vector for gene therapy is the retroviral vector. A recombinant retroviral vector may contain the following parts: an intact 5' LTR from an appropriate retrovirus, such as MMTV, followed by DNA containing the
30 retroviral packaging signal sequence; the insulator element placed between an LCR and the promoter of a transcription unit containing the gene to be introduced into a specific cell for replacement gene therapy; a selectable gene as described below; and a 3' LTR which contains a deletion in the viral enhancer region, or deletions
35 in both the viral enhancer and promoter regions. The selectable gene may or may

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not have a 5' promoter that is active in the packaging cell line, as well as in the transfected cell.

The recombinant retroviral vector DNA can be transfected into the amphotrophic packaging cell line Ψ -AM (see Cone, R. and Mulligan, R., 1984, Proc. Natl. Acad. Sci. USA, 81:6349) or other packaging cell lines which are capable of producing high titer stocks of helper-free recombinant retroviruses. After transfection, the packaging cell line is selected for resistance to G418, present at appropriate concentration in the growth medium.

Other chromatin insulator elements (e.g. both tissue-specific and non-specific) may be used in the constructs of the present invention, either by cloning and isolating eukaryotic constitutive hypersensitive sites having sequences similar to the chicken and human insulator elements disclosed herein, or by using other sequences known or tested to be constitutive hypersensitive sites that function as insulator elements.

Examples of transfectable reporter genes that can be used in the present invention include those genes whose function is desired or needed to be expressed *in vivo* or *in vitro* in a given cell or tissue type. Genes having significance for genetic or acquired disorders are particularly appropriate for use in the constructs and methods of the invention. Genes that may be insulated from *cis*-acting regulatory sequences by the insulator elements of the present invention may be selected from, but are not limited to, both structural and non-structural genes, or subunits thereof, such as those which encode proteins and glycoproteins (e.g. factors, cytokines, lymphokines), enzymes (e.g. key enzymes in biosynthetic pathways), hormones, which perform normal physiological, biochemical, and biosynthetic functions in cells and tissues. Other useable genes are selectable antibiotic resistance genes (e.g. the neomycin phosphotransferase gene (Neo[®]) or the methotrexate-resistant dihydrofolate reductase (dhfr) gene) or drug resistance genes (e.g. the multi-drug resistance (MDR) genes), and the like. Further, the genes may encode a precursor of a particular protein, or the like, which is modified intracellularly after translation to yield the molecule of interest. Further examples of genes to be used in the invention may include, but are not limited to,

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erythroid cell-specific genes, B-lymphocyte-specific genes, T-lymphocyte-specific genes, adenosine deaminase (ADA)-encoding genes, blood clotting factor-encoding genes, ion and transport channel-encoding genes, growth factor receptor- and hormone receptor-encoding genes, growth factor- and hormone-encoding genes, insulin-encoding genes, transcription factor-encoding genes, protooncogenes, cell cycle-regulating genes, nuclear and cytoplasmic structure-encoding genes, and enzyme-encoding genes.

The present invention is also applicable to targeting tumor or malignant cells with the insulator element-containing constructs carrying genes encoding toxins or toxoids, e.g. diphtheria toxoid and the like, to kill or otherwise damage and destroy the targeted cells. In addition, newly-cloned and isolated genes may be suitable candidates for use as reporter genes in the present invention.

Examples of eukaryotic promoters suitable for use in the invention are may include, but are not limited to, the thymidine kinase (TK) promoter, the alpha globin, beta globin, and gamma globin promoters, the human or mouse metallothionein promoter, the SV40 promoter, retroviral promoters, cytomegalovirus (CMV) promoter, and the like. The promoter normally associated with a particular structural gene which encodes the protein of interest is often desirable, but is not mandatory. Accordingly, promoters may be autologous or heterologous. Suitable promoters may be inducible, allowing induction of the expression of a gene upon addition of the appropriate inducer, or they may be non-inducible.

Further, a variety of eukaryotic enhancer elements may be used in the constructs of the invention. Like the promoters, the enhancer elements may be autologous or heterologous. Examples of suitable enhancers include, but are not limited to, erythroid-specific enhancers, (e.g. as described by Tuan, D. et al., 1985, "The β -like-globin" gene domain in human erythroid cells", Proc. Natl. Acad. Sci. USA, 82:6384-6388, and in U.S. Patent No. 5,126,260 to I.M. London et al.), the immunoglobulin enhancer, virus-specific enhancers, e.g. SV40 enhancers, or viral LTRs, pancreatic-specific enhancers, muscle-specific enhancers, fat cell-specific enhancers, liver specific enhancers, and neuron-specific enhancers.

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Many types of cells and cell lines (e.g. primary cell lines or established cell lines) and tissues are capable of being stably transfected by or receiving the constructs of the invention. Examples of cells that may be used include, but are not limited to, stem cells, B lymphocytes, T lymphocytes, macrophages, other white blood lymphocytes (e.g. myelocytes, macrophages, monocytes), immune system cells of different developmental stages, erythroid lineage cells, pancreatic cells, lung cells, muscle cells, liver cells, fat cells, neuronal cells, glial cells, other brain cells, transformed cells of various cell lineages corresponding to normal cell counterparts (e.g. K562, HEL, HL60, and MEL cells), and established or otherwise transformed cells lines derived from all of the foregoing. In addition, the constructs of the present invention may be transferred by various means directly into tissues, where they would stably integrate into the cells comprising the tissues. Further, the constructs containing the insulator elements of the invention can be introduced into primary cells at various stages of development, including the embryonic and fetal stages, so as to effect gene therapy at early stages of development.

As presented above, the constructs of the invention may contain one or more genes whose functional expression is buffered to different extents by the strategic placement of the insulator element in the construct. Using the constructs of the invention, two genes may be co-transfected into a cell or tissue type and their levels of expression can be regulated independently as a result of the positioning of the insulator element. For example, as shown in Fig. 4 and described further hereinbelow, placement of the insulator element between the LCR and the gamma-neo gene segment, and upstream of and flanking the LCR and the TK-hygromycin gene segment in the same construct, resulted in transfected colonies which had greater resistance to hygromycin than to neomycin. Such a construct demonstrates the importance of the position of the insulator element in the construct in relation to the LCR or enhancer and the gene of interest, and allows for the enhanced expression of one gene and the insulation (i.e. decreased or limited expression) of another gene also carried in the construct. One skilled in the art will further appreciate that differential expression of the products or

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components of the products of transfected genes in the constructs of the invention is useful in gene transfer and therapy studies.

5 In another embodiment of the invention, the constructs may be designed to contain genes encoding two subunits or components of a single protein so that each chain could be expressed from the same plasmid. For example, some proteins such as growth factors, growth factor receptors, blood clotting factors, and hormones are frequently comprised of two chains or subunits (e.g. α and β) which associate to form the functional molecule. In this embodiment, the gene coding for one chain or subunit of the molecule can be positioned in the plasmid or vector in
10 conjunction with the insulator elements and specific promoter and enhancer elements (or heterologous promoter and enhancer, if desired), and the gene coding for the other chain or subunit can be positioned in the same plasmid or vector in conjunction with its insulator, promoter, and enhancer elements. The plasmid or
15 vector containing the dual chain-encoding genes with their appropriately-positioned insulator elements can be transfected into cells to allow for the expression of a complete, two-chained molecule from the incorporated plasmid DNA, with each chain being regulated independently and with the copy numbers remaining the same.

20 Also contemplated by the invention is a kit or kits containing insulator constructs in which the insulator elements of the invention are provided in a DNA receivable vector or plasmid that contains or can be readily adapted by the user to contain the appropriate DNA elements for proper expression of a gene or
25 genes of interest. For example, the vector or plasmid may contain one or more insulator elements on either end of a stretch of vector or plasmid DNA containing either a polylinker or a *NorI* restriction enzyme insertion site for receiving a variety of genetic elements for proper expression of the gene or genes of interest. The
30 insulator element-containing plasmids or vectors of the kit may contain insulator elements, enhancers or LCRs, a transcription unit, and the gene or genes of interest may be inserted between the insulators, as desired. Alternatively, the constructs of the kit may contain some or all of the necessary genetic elements for
35 proper gene expression, or combinations of these, and the remaining genetic

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elements may be provided and readily inserted by the user, preferably between the insulator elements in the construct. The insulator element-containing plasmids or vectors may be provided in containers (e.g. sealable test tubes and the like) in the kit and are provided in the appropriate storage buffer or medium for use and for stable, long-term storage. The medium may contain stabilizers and may require
5 dilution by the user. Further, the constructs may be provided in a freeze-dried form and may require reconstitution in the appropriate buffer or medium prior to use.

10 The Constitutive Hypersensitive Site is GC-rich

Fig. 2 reveals the sequence of the isolated 242 bp sequence of DNA (Seq ID No:1) which comprises the chicken 5' constitutive hypersensitive site or insulator element as defined by the invention. In the exemplary constructs
15 demonstrating the insulating function of the insulator element, the core 242 bp sequence comprising insulator element is contained within a 1.2 kb segment of DNA comprising the constitutive hypersensitive site at the 5' end of the chicken beta-globin locus. The 1.2 kb *SacI-SspI* fragment containing the insulator element was isolated by restricting the plasmid pCBGC (described by Reitman, M. and
20 Felsenfeld, G. 1990, Mol. Cell. Biol., 10:2774-2786) with *HindIII*. The isolated 242 bp constitutive hypersensitive site, and portions thereof, possess demonstrable insulator activity, as does the larger isolated 1.2 kb DNA segment containing the 242 bp segment; both segments of DNA are useful in the invention. It is noted
25 that the 242 bp sequence possesses significant insulator activity; however, other DNA sequences within the 1.2 kb *SacI-SspI* fragment may also influence or augment the insulating activity of the core 242 bp sequence comprising the insulator element of the invention.

30 Sequence analysis of the constitutive hypersensitive site or insulator element of the invention shows that the insulator element is relatively GC-rich (69%). In addition, the extremely high frequency of the C_pG dinucleotide sequence (i.e. 21 times in 242 bp) in the sequence is unusual for a vertebrate sequence.
35 Such a high density of the C_pG dinucleotide sequence may serve to identify

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homologous DNA sequences as insulator elements in accordance with the present invention. Further, the 242 bp insulator element sequence is comprised of a number of repeating "CAG" and "CCG" sequences throughout its length. The CAG repeat units have been implicated as a source of mutations for various diseases. A strong correlation may exist between an increased number of copies of
5 either or both the CAG and the CCG repeating units and a mutant or transformed state. Thus, the number of CAG and/or CCG repeats contained within a given sequence may be critical in determining the normal form versus the mutant form of that sequence or of a given gene.

10 In contrast, both the previously-described chicken lysozyme "A" element and the *Drosophila* scs have been shown to be relatively AT-rich (73%), (Farkas, G. and Udvardy, A. (1992), "Sequence of scs and scs' *Drosophila* DNA fragments with boundary function in the control of gene expression", Nucl. Acids Res., 20:2604). This finding indicates that the chromatin insulator element of the
15 invention is very distinct from the previously-described "A" element and scs.

Characterization of the Insulator Element

20 In the present invention, the chicken beta-globin locus was used to isolate a 5' constitutive hypersensitive site and to fully characterize and show actual functionality of this site as a chromatin insulating element in a mammalian system. In accordance with the invention, plasmids containing the chicken constitutive hypersensitive site have been demonstrated experimentally to insulate a reporter
25 gene from the influence of a nearby LCR when the constitutive hypersensitive site is positioned in the plasmid between the LCR and the promoter of the reporter gene and the plasmid is transfected into a mammalian cell such as the erythroleukemia cell line K562 (available from the American Type Cell Culture
30 Collection, Rockville, MD, Accession No. ATCC CCL 243)

In particular, the present invention utilized exemplary constructs containing, as a reporter gene segment, the G418-resistance gene (also called the neomycin, or "neo", resistance gene) which codes for resistance to the antibiotic
35 neomycin or G418, driven by the gamma-globin gene promoter to demonstrate the

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insulating activity of the insulator element. Normal eukaryotic cells do not grow or persist in the presence of G418, unless they have been made resistant to the antibiotic, either by transfecting in the gene for G418 resistance or by some other means. After transfecting the construct into human cells, the number of clonal cell colonies that were able to survive and grow in the presence of G418 in the culture medium were counted. Individual G418-resistant cell clones can be isolated and expanded to select for clones which express the gene at high levels. Also used as a co-reporter gene in the constructs of the invention was a gene segment carrying the thymidine kinase promoter and the hygromycin resistance gene which codes for resistance to the antibiotic hygromycin.

Moon and Ley (1990, Proc. Natl. Acad. Sci. USA, 87:7693-7697) disclosed that the presence of a mouse or human LCR (i.e. a 5' HS2 enhancer element) increased the number of G418 resistant K562 cell colonies by 30 to 100 fold. This agrees with the significant increase in the level of reporter gene transcript that the LCR confers in such erythroid-specific cells. One of the advantages of using the colony assay of the invention, rather than isolating transfected cells with a selection marker first, and then assaying the level of reporter mRNA or an enzyme activity such as CAT (chloramphenicol acetyltransferase) afterward, is that there is no prior selection bias for integration into a region of open chromatin. This is true even if the selection marker is co-transfected with the reporter gene, since the reporter gene and the selection marker gene often integrate into the same region of chromatin in tandem arrays.

To test whether the constitutive hypersensitive site of the chicken beta-globin locus was able to insulate a reporter gene from a nearby, strongly active LCR, constructs as shown in Fig. 3A were stably transfected into the genome of a human erythroleukemia cell line K562. If the constitutive hypersensitive site insulates the γ -neomycin gene from the LCR, a significant decrease in the number of neomycin resistant colonies would be observed. As demonstrated in Fig. 3A, the presence of one copy of the chicken constitutive hypersensitive site on either side of the G418 resistance reporter gene (pJC5-4) decreased the number of G418 resistant colonies by about 9 to 10 fold as compared

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with the control plasmid pJC3-4 (i.e. the 2.3 kb *HindIII-HindIII* fragment from phage λ DNA). In addition, the presence of two copies of the constitutive hypersensitive site on either side of the reporter gene (pJC13-1) further decreased the number of G418 resistant colonies by about 30-fold. These results demonstrated that the constitutive hypersensitive site successfully insulated the
5 gamma-globin promoter and the G418 resistance reporter gene segment (i.e. the γ -neo reporter gene) from the action of LCR.

To determine if the decrease in the number of G418 resistant colonies resulted from an effect on the position of integration or from the copy
10 number of the integrated gamma-neo genes in the genome of the host cells, a second selection marker DNA segment or co-reporter gene segment was positioned next to the LCR as an internal control as shown in Fig. 3B. This second selection marker DNA segment comprised the *Herpes simplex* virus (HSV) thymidine kinase ("TK") promoter which controlled the transcription of the hygromycin gene, and
15 was denoted "TK-hygromycin", abbreviated "TK-HYG". Plasmids containing the constructs as shown in Fig. 3B were stably transfected into human K562 cells. Neomycin-resistant colonies and hygromycin-resistant colonies were subsequently counted. The ratio of neomycin- to hygromycin-resistant colonies for each
20 construct is shown in Fig. 3B. The number of hygromycin-resistant colonies counted for all four of the constructs used were very similar. Similar to the constructs shown in Fig. 3A, the presence of one copy of the chicken 5' constitutive hypersensitive site on either side of the reporter gene segment led to
25 about a 7 to 8-fold decrease in expression of the reporter gene (5-4Neo/Hyg versus 3-4Neo/Hyg). In addition, the presence of two copies of the chicken 5' constitutive hypersensitive site on either side of the reporter segment led to about a 20-fold decrease in expression (13-1Neo/Hyg versus 3-4Neo/Hyg). These data
30 confirm the results shown in Fig. 3A and rule out the possibility that the position of integration or the copy number is responsible for the insulating effect observed in Fig. 3A. Fig. 3A and 3B also point out that the chicken 5' constitutive hypersensitive site (i.e. insulator element) effectively insulates the reporter gene from the LCR when the insulator element is placed between the LCR and the
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reporter gene segment.

Further, the deletion of the LCR from the constructs shown in Fig. 3 led to an approximately 40-fold decrease in the number of neomycin resistant colonies. Taken together, these data indicate that the chicken constitutive hypersensitive site effectively but incompletely (7 to 10 fold instead of 40 fold) insulates the reporter gene from the LCR when only one insulator site is placed between the reporter gene and the LCR, but almost completely when two insulator sites are placed between the reporter gene and the LCR (20 to 30 fold).

To demonstrate the insulation effect at the mRNA level, the clones isolated after stable transfection with the two constructs (3-4Neo/Hyg and 5-4Neo/Hyg) shown in Figure 3B were characterized. Clonal populations of hygromycin-resistant cells were selected for growth in hygromycin, mRNA was isolated by conventional methods, and neomycin and hygromycin mRNA was analyzed by RNase protection assays as shown in Fig. 4A and 4B. Lanes 1-4 each represent a discrete clone of cells (i.e. clones 1-4) which was isolated from the agar selection medium. As expected, when control λ phage DNA was placed between the LCR and the gamma-neomycin gene (3-4Neo/Hyg), the gamma-neomycin gene and the TK-hygromycin gene were expressed in roughly comparative levels (clone 1). Clone 2, which was also transfected with the 3-4Neo/Hyg plasmid, contained a lower copy number. However, when one copy of the insulator element of the invention was positioned between the LCR and the gamma-neomycin gene (5-4Neo/Hyg), hygromycin gene expression was virtually unaffected, while the neomycin mRNA was either completely absent (clones 3 and 4) or decreased about 2-fold (clone 5) relative to the hygromycin mRNA level. Thus, it appeared that insulation with one copy of the insulator element is complete at the level of the mRNA, but was somewhat leaky (e.g. clone 5) depending on the site of integration of the transfected DNA. It was further discovered using Southern blot analysis that clone 5 had seven copies of the transfected DNA, while clones 1-4 had only one to three copies of the integrated reporter gene construct. Thus, it is possible that when there are numerous copies of the DNA integrated into the transfected cell DNA, some of the copies may not be completely insulated.

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In accordance with the present invention, only one chromatin insulator element exists at the 5' boundary of the chicken β -globin domain and yet one chromatin insulator placed in the 5-4Neo/Hyg construct can be leaky depending on the site of integration and perhaps due to high copy number (e.g. clone 5, Figure 4A). Several possible explanations are proposed for this finding:

5 i). there may be one or more elements, in addition to the constitutive hypersensitive site of the invention, in the 5' boundary region of chromatin that are required for a full insulator activity; ii). the 5' chromatin insulator element may interact with an as yet to be identified complementary chromatin insulator at the 3' boundary of the β -globin domain for full insulator activity; iii). the distances
10 between the LCR, the chromatin insulator element, and the reporter gene promoter may be more critical and may be too close in the transfected DNA; and iv). during the integration of the transfected DNA, the LCR-promoter complex may have
15 formed before the insulator complex had formed. However, in spite of the foregoing explanations, it appears that whatever may be lacking with only one copy of the chromatin insulator element used in the constructs of the invention, having two copies of the chromatin insulator element is compensatory and results in almost complete insulation.

20 The chicken-derived insulator element of the invention functions with regulatory elements other than those of chicken cells and in species more divergent than chickens. For example, experiments were conducted which employed P-element mediated transformation to introduce into transgenic Drosophila plasmid
25 constructs containing the Drosophila *white* minigene flanked with two copies of the insulator element (J. H. Chung et al. (1993). "A 5' element of the chicken β -globin domain serves as an insulator in human erythroid cells and protects against position effect in Drosophila". Cell, 74:505-514). The plasmid constructs were similar to
30 those depicted in Fig. 3B, except that the γ -neo gene was replaced by the Drosophila *white* minigene flanked either by two copies of the insulator element, or, as a control, by λ DNA. The results showed that the expression of the *white* minigene of the fruit fly was protected against position effects in independently-generated transgenic fruit fly lines. The success of the isolated insulator element in
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preventing position effects in Drosophila eye cells showed that the insulator has a broad range of function, beyond the exemplary erythroid cells. Thus, the insulator element is capable of functional utility across a wide evolutionary spectrum.

Directionality of Insulation

5 The main operational difference between an insulator and a classic silencer is the directionality of insulation. In accordance with the functional assays used to demonstrate insulation as presented, the insulator element exerted its effect when it was placed between the LCR and the reporter gene segment, but not when
10 it flanked them. By contrast, a classic silencer suppresses gene expression regardless of its position (Renkawitz, R. 1990, "Transcriptional repression in eukaryotes", TIG, 6:162-197; Wada-Kiyama, Y. et al., 1992, "The ϵ -globin Gene Silencer. Characterization by *in vitro* transcription", J. Biol. Chem., 267:11532-
15 11538).

 To demonstrate a specific directionality in the functioning of the eukaryotic insulator elements of the invention, K562 cells were stably transfected with the constructs shown in Figure 5A, in which γ -neomycin gene segment was flanked by either the chicken hypersensitive site (pJC16) or the control λ phage
20 DNA (pJC17). Fig. 5A shows that the number of neomycin-resistant colonies did not change significantly when the γ -neomycin gene segment was flanked by the constitutive hypersensitive sites. Similar results were seen when the constructs shown in Fig. 5B were used. Fig. 5B shows that when the LCR/ γ -neomycin gene
25 was flanked either by the constitutive hypersensitive site of the invention or by λ phage DNA, there was a minimal increase in the number of neomycin resistant colonies resulting from transfection with the constructs carrying the insulator element DNA segment. The data presented indicate that the constitutive
30 hypersensitive site works in a directional manner in the constructs. Further, because it does not perturb gene expression significantly on its own, it is a "pure" chromatin insulator, as opposed to a classic silencer which does not show directionality.

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Chromatin Insulator Element Blocks the LCR From Disrupting the Nucleosome in the Promoter Region

Data from naturally occurring beta thalassemias, transgenic mice and transfection studies indicate that the LCRs play a role in displacing the nucleosome in the region of the promoters of the genes in the β -globin domain and in forming an active transcriptional complex (reviewed by Felsenfeld, G. (1992). "Chromatin as an essential part of the transcriptional mechanism". Nature, 355:219-224). The state of the promoter nucleosome was examined to determine whether, in the presence of the chromatin insulator element, the promoter nucleosome was undisrupted and the transcriptional complex was not formed, or whether the promoter nucleosome was displaced, but the transcriptional complex formed was inactive.

In the Southern blot shown in Figure 6A, the presence of the promoter nucleosome was assessed by examining the accessibility of the *ApaI* restriction enzyme site in the γ -neomycin gene promoter to *ApaI* added to intact nuclei of K562 cells transfected with the constructs shown in Figure 3B. After nuclei digestion with *ApaI*, genomic DNA was isolated and cut with *XbaI* and *BglII* to generate the parental DNA fragments. If, as in HeLa cells where the γ -globin promoters and the LCRs are inactive, the nucleosome over the γ -globin gene promoter is undisrupted, the *ApaI* site would be inaccessible, and thus, the *ApaI* enzyme will not cut. If the nucleosome is disrupted, the *ApaI* site would be accessible to the *ApaI* enzyme and the enzyme would cut at its specific site to generate a 570 bp fragment (*) and a 883 bp fragment (*) for the γ -neomycin and the endogenous γ -globin promoters, respectively. These fragment would be observed when the Southern blot containing K562 genomic DNA is probed with a probe comprising the γ promoter as designated in Figure 6B.

Other work in the inventors' laboratory has shown that in K562 cells which express the γ and ϵ -globin genes, the nucleosomes over the promoters are displaced. Because the promoter of the γ -neomycin gene is isolated from the endogenous γ -globin gene, and the promoters of the endogenous γ and ϵ -globin genes are identical, the accessibility of the *ApaI* site in the γ -neomycin gene can be

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compared with that in the two endogenous γ -globin gene promoters on the same Southern blot.

As demonstrated in Figure 6A, the *ApaI* site in the endogenous γ -globin promoters ($\gamma^G\gamma$) cut the K562 DNA with 16% to 24% efficiency for the three constructs, while the *ApaI* site in the γ -neomycin promoter (γ -neo*) cut only
5 in construct 3-4Neo/Hyg, which contains no insulator element. Further, the parental γ -Neo band decreased significantly (i.e. 22%) only in construct 3-4Neo/Hyg, which has no chromatin insulator (lane 2). Cutting by *ApaI* at the γ -neomycin promoter (γ -neo*) was not visible when the LCR was blocked by the
10 chromatin insulator element (lanes 5 and 8) in constructs 13-1Neo/Hyg and 5-4Neo/Hyg. In lanes 3, 6, and 9, the genomic DNA was isolated prior to cutting with *ApaI*.

The data in Fig. 6A and 6B indicate that the nucleosome over the γ -neomycin promoter was undisplaced when the LCR was blocked by the chromatin
15 insulator element (as in constructs 13-1Neo/Hyg and 5-4Neo/Hyg), but was displaced when the LCR was not blocked by the insulator element (as in construct 3-4Neo/Hyg). Thus, it is clear that the presence of the insulator element between the LCR and the γ -neomycin gene segment interfered with the cutting by *ApaI*, and
20 therefore prevented the formation of a nucleosome-free region at the promoter. In addition, it is also likely that the insulator prevented the formation of an active transcriptional complex in the promoter region of the chromatin.

25 Mechanism of Insulation

The present invention also provides mechanistic models to demonstrate how the eukaryotic chromatin insulator element may block the action of an LCR in human cells. Studies of naturally-occurring beta thalassemias and
30 studies using transgenic mice have indicated that enhancers or LCRs play a critical role in displacing or disrupting the nucleosomal structure at the promoter of the genes in the beta-globin domain and in forming an active transcriptional complex at the promoter. While the nucleosomal structure at the promoter is disrupted in all
35 expressing genes, there is no such correlation for inactive genes. In other words,

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it is possible to have nucleosomal disruption and yet have no gene expression.

The findings herein have suggested that the chromatin insulator element prevents the LCR from disrupting or displacing the nucleosome at the promoter, either directly or indirectly, thereby disrupting the formation of the transcriptional complex at the promoter.

5 Exactly how the insulator accomplishes the task of maintaining the integrity of the nucleosome at the promoter and blocking the LCR from forming a transcription complex is not clear. However, three models can be proposed (Fig. 7). In model A of Fig. 7, the LCR and its bound nucleoprotein factors may track
10 along the DNA and "loop out" the intervening sequences (Muller, H. Sogo, J.M. and Schaffner, W. (1989). "An enhancer stimulates transcription in trans when attached to the promoter via a protein bridge". Cell, 58:767-777).
in search of the target promoter; when the LCR protein complex reaches the target
15 promoter, it would stop. In this model, the chromatin insulator element poses a block to the progress of the LCR complex and would prevent its reaching the target promoter. In model B, the LCR complex may "skip" along the DNA, again
looping out the intervening sequences. The insulator element in model B somehow
20 reduces the mobility of the LCR complex, perhaps by immobilizing the intervening DNA and strengthening the chromatin to allow resistance to LCR activity. In
model C, the 5' chromatin insulator element forms a complex with a 3' chromatin
insulator and also forms a loop that excludes the LCR. Such an insulator-insulator
25 complex might reduce the mobility of the LCR by immobilizing the intervening sequence or topologically isolating the LCR.

Application of the Chromatin Insulator Element for Use in Gene Therapy and Gene Transfer

30 The eukaryotic insulator element of the invention provides the first "pure" chromatin insulator that works in mammalian, e.g. human, cells. Accordingly, the insulator element has important practical implications for improved gene therapy of human genetic diseases which are frequently
35 characterized by deficient expression of normal structural genes or expression of abnormal structural genes in particular cell types, e.g. erythroid cells,

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lymphocytes, islet cells, to name only a few. Examples of pathologies involving the erythroid lineage are: sickle cell disease and other hemaglobinopathies, thalassemias, enzyme deficiency diseases (e.g. glucose-6-phosphate dehydrogenase deficiency and pyruvate kinase deficiency). The insulator-containing constructs described herein can be introduced into abnormal cells and tissues to compensate
5 for production of an abnormal protein or a protein which is deficient or missing altogether in the cell.

One of the main problems of current gene therapy techniques is the gradual loss of expression of the transfected gene, perhaps due to the repressive
10 influence of the DNA sequences which surround the integration site of the transfected gene (Palmer, T.D., Rosman, G.J., Osborne, W.R. and Miller, D. (1991). "Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes". Proc. Natl. Acad. Sci. USA, 88:1330-
15 1334; Scharfmann, R., Axelrod, J.H. and Verma, I.M. (1991). "Long-term in vivo expression of retrovirus-mediated gene transfer in mouse fibroblast implants". Proc. Natl. Acad. Sci. USA, 88:4626-4630). By insulating a gene to be transfected with the insulator element of the present invention, the gene could be maintained in an active state.

20 Another problem of gene therapy is the danger that the enhancer or regulatory element of the transfected gene, when integrated in close proximity to an oncogene, may actually promote tumor formation. Again, by insulating the transfected gene with the chromatin insulator element of the invention, the
25 enhancer or regulatory elements of the transfected gene may be prevented from influencing the expression of critical endogenous genes whose activities may be harmful or detrimental to the host. Thus, it is critical that the insulator by itself does not perturb the expression of nearby genes. Similarly, the insulator element
30 should be useful for making transgenic animal expressing certain gene products at various levels and/or at particular times in development. Further, the constructs of the invention, used in the production of transgenic animals, would circumvent the problems encountered when DNA introduced into the animal cells or embryos becomes integrated in nonexpressing or silent areas of the chromatin.
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The insulator element also promises to be a useful tool in gene regulation studies and in the production of stably transfected cell lines. Most frequently, integration of a transfected gene or construct into host cell genome occurs at random. Because the expression of a stably transfected gene is influenced by adjacent regulatory elements near the site of gene integration, insulating the transfected gene with the insulator elements of the present invention eliminates the variability that is caused by cell-to-cell differences in integration position and in the random sites of integration. Thus, genes insulated with the chromatin insulator element of the invention will be free of position dependence and will be protected from the regulatory elements of the selection marker gene in the case of the stably transfected cell lines. This should obviate the need for numerous founder lines of clonal cell lines.

In general, gene therapy techniques for a genetic disorder characterized by deficient or abnormal expression of a protein, or by the complete absence of a gene, may be carried out as follows: bone marrow from a patient is removed (e.g. by aspiration under sterile conditions) and the bone marrow cells are incubated with the vector with its DNA construct comprising one or more insulator elements, an enhancer, and a transcription unit which comprises a promoter and a gene which encodes the normal protein or precursor thereof, or contains a normal version of a gene, under conditions which allow the vector with its DNA construct to be incorporated into the cells. The treated bone marrow cells are then reinfused into the patient. The procedure can be repeated several times in order to increase the total number of marrow cells into which the normal gene has been inserted. The constructs of the invention can be designed to contain insulator elements and a transcriptional enhancer element that is specific for the transcription of a particular transcription unit. For example, a transcription unit encoding a normal erythroid protein, or precursor thereof, can be used and the insulator elements can be positioned so as to insulate the transcription of the erythroid-specific gene. In gene therapies of human hemoglobin disorders of the beta chain of hemoglobin (i.e. where the synthesis of a normal beta-globin chain is deficient or where an abnormal chain is synthesized), a vector-DNA construct containing the insulator

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elements and a transcription unit encoding beta-globin and the beta-globin transcriptional enhancer is incorporated into bone marrow cells. In this exemplary case, treatment of bone marrow cells will result in the insulated incorporation of the vector-DNA construct into erythroid precursor cells and hematopoietic stem cells, thereby allowing expression of the globin genes, free from any *cis*-acting regulatory influences of the surrounding DNA.

In another embodiment, the constructs of the invention may be used to transfect a gene or genes into cells which lack a particular gene or genes, or which fail to produce a particular enzyme, protein, or critical cellular component. The transfected cells containing the "insulated" gene or genes can stably express the missing expression product in the absence of any influences from the surrounding DNA at the site of integration. Stably transfected cells may be administered to patients, either via transplanting transfected cells or seeding the transfected cells into an appropriate tissue or organ.

The constructs may be administered in the form of a pharmaceutical preparation or composition containing a pharmaceutically acceptable carrier and a physiological excipient, in which preparation the vector may be a viral vector construct, or the like, to target the cells, tissues, or organs of interest. The composition may be formed by dispersing the components in a suitable pharmaceutically-acceptable liquid or solution such as sterile physiological saline or other injectable aqueous liquids. The composition may be administered parenterally, including subcutaneous, intravenous, intramuscular, or intrasternal routes of injection. For injectable administration, the composition is in sterile solution or suspension or may be emulsified in pharmaceutically- and physiologically-acceptable aqueous or oleaginous vehicles, which may contain preservatives, stabilizers, and material for rendering the solution or suspension isotonic with body fluids (i.e. blood) of the recipient. Excipients suitable for use are water, phosphate buffered saline, pH 7.4, 0.15 M aqueous sodium chloride solution, dextrose, glycerol, dilute ethanol, and the like, and mixtures thereof.

As but one example, cystic fibrosis is an hereditary disease of the exocrine glands, usually developing during early childhood and affecting mainly the

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pancreas, respiratory system and sweat glands. It is characterized by the production of abnormally viscous mucous by the affected glands, usually resulting in chronic respiratory infections and impaired pancreatic function. At the molecular level, the disease is known to be caused by the lack of the gene coding for the chloride ion channel in cells. The cystic fibrosis gene is a good candidate to use in the constructs of the invention to transfect target cells of the appropriate organs or glands such that when the gene is expressed in those cells lacking the gene (e.g. pancreatic cells, lung cells, sweat gland cells), the cells will have functional chloride channels; the expression of the gene coding for the chloride channel will be insulated from the effects of surrounding DNA and the transfected gene, and the associated DNA in the construct will not influence any sequences in *cis* in the host DNA.

15 **EXAMPLES**

The examples herein are meant to exemplify the various aspects of carrying out the invention and are not intended to limit the invention in any way.

20 **EXAMPLE 1**

Construction of plasmids for DNA transfection

The plasmid constructs shown in Figure 3A were constructed as follows: the plasmids pJC3-4, pJC5-4, and pJC13-1 were constructed by initially inserting the 1.1 kb *EcoRI-EcoRI* fragment (Moon, A.M. and Ley, T.J. (1990). "Conservation of the primary structure, organization, and function of the human and mouse β -globin locus-activating regions". Proc. Natl. Acad. Sci. USA, 87:7693-7697) containing the mouse 5'HS2 (LCR) into the *EcoRI* site of the vector pGEM-4Z (Promega). The 2.7 kb *BamHI-BamHI* fragment containing the human γ -globin promoter linked to the neomycin (G418) resistance gene (Moon and Ley, 1990, Proc. Natl. Acad. Sci. USA, 87:7693-7697) was then inserted into the *BamHI* site of this plasmid.

To make pJC3-4, the 2.3 kb *HindIII-HindIII* fragment from the λ phage DNA was blunted with Klenow and cloned into the blunted *SacI* site and

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*Xba*I site using an *Xba*I linker. The plasmid pJC5-4 was made similarly by inserting the 1.2 kb *Sac*I-*Ssp*I fragment, which was isolated by *Hind*III digestion of pCBGC (Reitman, M. and Felsenfeld, G. (1990). "Developmental regulation of topoisomerase II sites and DNase I-hypersensitive sites in the chicken β -globin locus". Mol. Cell. Biol., 10:2774-2786) into the *Sac*I and *Xba*I sites after ligating the corresponding linkers. The isolated 1.2 kb fragment contained the chicken constitutive hypersensitive site (5'HS4), previously unknown or unidentified as an insulator. The plasmid pJC13-1 was made by inserting one 1.2 kb *Sac*I-*Ssp*I fragment (i.e. the chicken 5'HS4-containing fragment) into the *Kpn*I site, one 1.2 kb *Sac*I-*Ssp*I fragment into the *Sac*I site, and two 1.2 kb *Sac*I-*Ssp*I fragments into the *Xba*I site.

In order to create the constructs shown in Figure 3B, plasmid pJC78 was first created by inserting the HSV TK-hygromycin resistance gene from pHyg (Sugden, B., Marsh, K., and Yates, J., 1985, "A vector that replicates as a plasmid and can be efficiently selected in B lymphoblasts transformed by Epstein-Barr virus", Mol. Cell. Biol., 5:410-413) into the *Sac*I-*Bam*HI site and the mouse 5'HS (LCR) into the *Eco*RI site of vector pGEM-4Z. The *Ase*I-*Xmn*I fragment containing the 5'HS2 and the TK-hygromycin segment was isolated from pJC78 and ligated to plasmids pJC3-4, pJC5-4, and pJC13-1 which had been cut with *Nde*I and *Sal*I to create 3-4Neo/Hyg, 5-4Neo/Hyg, and 13-1Neo/Hyg, respectively. The ligated products were isolated from low melting agarose gels.

The plasmid pJC16 was made by removing the *Eco*RI-*Eco*RI fragment containing the mouse 5'HS2 from pJC5-4. The plasmid p17 was constructed by replacing the *Sac*I-*Sac*I and the *Xba*I-*Xba*I inserts of pJC16 with a 950 bp *Eco*RI-*Hind*III fragment from λ phage DNA ligated to either *Sac*I or to *Xba*I linkers, respectively, after blunting with Klenow. Plasmids pJC19 and pJC20 were made by inserting the 1.1 kb fragment containing the mouse 5'HS2 into the *Kpn*I site of pJC16 and pJC17, respectively, after a *Kpn*I linker was ligated thereto.

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EXAMPLE 2**Transfection of human cells and colony assays**

In a typical experiment, 10^7 mid-log phase K562 erythroleukemia cells (Ney, P.A., Sorrentino, B.P., McDonagh, K.T. and Nienhuis, A.W. (1990). "Tandem AP-1 binding sites within the human β -globin dominant control region as an inducible enhancer in erythroid cells". Genes & Dev., 4:993-1006) were harvested and washed once with PBS (phosphate buffered saline, about pH 7.4) prior to resuspension in 0.5 mL of cold PBS. 0.25 μ g of linearized DNA was added and mixed. After 10 minutes on ice, the cells were electroschocked using the BioRad Gene Pulser at 200V and 960 μ F. After 15 minutes on ice, the transfected cells were transferred to 35 mL of IMEM (Iscove's minimal essential medium) supplemented to contain 10% fetal calf serum. For constructs pJC16 and pJC17, 10 μ g of linearized DNA was used.

To generate neomycin (G418) or hygromycin resistant colonies, 3 mL of transfected cells (about 1×10^6 cells) were diluted 1:10 in IMEM supplemented to contain 10% fetal calf serum, 0.3% cell culture agar (Sigma), and about 500 to 1000 μ g/ml of active G418 (Gibco) or 300 units/ml of hygromycin (Sigma). The transfected cells were then plated in petri dishes at a density of about 1×10^5 /mL one to two days after transfection. Discrete, macroscopic neomycin and hygromycin resistant colonies of cells were counted two to three weeks after selection.

EXAMPLE 3**RNase Protection Assay**

Clonal cells from the experiment described in Figure 3B were isolated and grown in hygromycin. RNA was isolated from late log-phase cells with RNazol (Cinna/Biotechx) and RNase protection assays were performed on 30 μ g of RNA using the RPA II kit (Ambion). The probe for the γ -neomycin resistance gene RNA was derived from a 457 bp *Bam*HI-*Alw*NI fragment in plasmid γ -Neo (Moon, A.M. and Ley, T.J. (1990). "Conservation of the primary structure, organization, and function of the human and mouse β -globin locus-

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activating regions". Proc. Natl. Acad. Sci. USA, 87:7693-7697) containing the γ -globin promoter which was then cloned into the *Bam*HI-*A*lvNI site of pBluescript II SK+ (Stratagene). The RNA probe was synthesized with the mRNA capping kit (Stratagene) in the presence of [α - 32 P]UTP and T7 RNA polymerase. The probe protected a 143 bp band specific for the neomycin resistance gene RNA. The probe for TK-hygromycin resistance gene RNA was derived from the 333 bp *Mlu*I-*Eco*RI fragment in pHyg (Sugden, B., Marsh, K., and Yates, J., 1985, "A vector that replicates as a plasmid and can be efficiently selected in B lymphoblasts transformed by Epstein-Barr virus", Mol. Cell. Biol., 5:410-413) which was cloned into the *Sma*I-*Eco*RI site of pBluescript II SK+ and linearized at the *Sma*I site within the hygromycin gene. The RNA probe was synthesized with the mRNA capping kit (Stratagene) in the presence of [α - 32 P]UTP and T3 RNA polymerase. The probe protected a 260 bp band specific for the hygromycin resistance gene RNA.

EXAMPLE 4

Nuclei Digestion with *Apa*I Restriction Endonuclease

Pooled cells representing about 100 to 200 clones from the experiment disclosed in Figure 3B were grown in hygromycin. Approximately 3×10^7 cells were centrifuged at about 2000 rpm and washed once with cold phosphate buffered saline. The cells were then resuspended in 400 μ L of lysis buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 0.2% Nonident P-40, and 5 mM dithiothreitol and immediately centrifuged in a TOMY MTX 150 microcentrifuge for 4 minutes at about 2000 rpm. The nuclear pellet was then washed once with a wash buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, and 5 mM dithiothreitol by spinning in the TOMY MTX 150 microcentrifuge for 2 minutes. The nuclear pellet was resuspended in 400 μ L of digestion buffer containing 100 units of *Apa*I, 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol and incubated at 30°C for 20 minutes. Genomic DNA was isolated by standard methods known to those skilled in the art and was digested to completion with *Bgl*II and *Xba*I. The

- 40 -

digested DNA (15 μ g) was analyzed by the standard Southern blot method and probed with a 335 bp *Bam*HI-*Hin*III fragment from the plasmid γ -Neo (Moon and Ley, 1990) containing the γ -globin promoter. The probe was labeled with [α -³²P]dCTP using the random-primed DNA labeling kit (Boehringer Mannheim). Southern blot hybridization was performed in QuikHyb rapid hybridization solution (Stratagene).

EXAMPLE 5

DNA Sequencing

The 1.2 kb *Sac*I-*Ssp*I fragment for pCBGC was cleaved with *Alu*I and *Taq*I and cloned into the *Sma*I and *Acc*I sites of pBluescript II SK+ (Stratagene), respectively. Using primers against T3 and T7 promoters, the fragments were sequenced with the Sequenase Version 2.0 kit (United States Biochemical) and linked together.

The contents of the patents and references contained herein are hereby incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to those skilled in the art that certain changes and modifications may be practiced without departing from the spirit and scope thereof, as described in the specification and as defined in the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS:

- (A) NAME: The Government of the United States of America, as represented by the Secretary, Department of Health and Human Services
- (B) STREET: National Institutes of Health, Office of Technology Transfer, Box 13, 6011 Executive Boulevard
- (C) CITY: Rockville
- (D) STATE OR PROVINCE: Maryland
- (E) COUNTRY: United States of America
- (F) POSTAL CODE: 20852

(ii) TITLE OF INVENTION: DNA sequence which acts as a chromatin insulator element to protect expressed genes from cis-acting regulatory sequences in mammalian cells

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy Disk
- (B) COMPUTER: IBM PC Compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: Wordperfect 5.1

(v) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE: 06-Apr-1994

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US/08/045,266
- (B) FILING DATE: 07-Apr-1993

(vii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Leslie A. Serunian
- (B) REGISTRATION NUMBER: 35,353
- (C) REFERENCE/DOCKET NUMBER: 2026-4049PCT

(viii) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (212) 758-4800
- (B) TELEFAX: (212) 751-6849

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 242 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(iii) HYPOTHETICAL: No

(vi) ORIGINAL SOURCE:

5 (A) ORGANISM:

(B) STRAIN:

(C) INDIVIDUAL ISOLATE:

(D) DEVELOPMENTAL STAGE:

(E) HAPLOTYPE:

(F) TISSUE TYPE:

(G) CELL TYPE:

(H) CELL LINE:

10 (I) ORGANELLE:

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION:

(C) IDENTIFICATION METHOD:

15 (D) OTHER INFORMATION: 242 base pair DNA
sequence comprising a portion of the
chicken 5' constitutive
hypersensitive site

(xi) SEQUENCE DESCRIPTIONS:SEQ ID NO:1:

AGGGACAGCC CCCCCCAA GCCCCAGGG ATGTAATTAC	40
GTCCCTCCCC CGCTAGGGCA GCAGCGAGCC GCCCGGGGCT	80
CCGGTCCGGT CCGGCGCTCC CCCGCATCCC CGAGCCGGCA	120
GCGTGCGGGG ACAGCCCGGG CACGGGGAAG GTGGCACGGG	160
ATCGCTTTCC TCTGAACGCT TCTCGCTGCT CTTTGAGCCT	200
25 GCAGACACCT GGGGGGATAC GGGGAAAAAA GCTTTAGGCT	240
GA	242

30

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WHAT IS CLAIMED IS:

1. An isolated DNA molecule comprising a eukaryotic 5' constitutive DNase I-hypersensitive site corresponding to the chicken 5' HS4 constitutive hypersensitive site; wherein the DNA molecule is a chromatin insulator and insulates the transcriptional expression of genes from one or more *cis*-acting regulatory sequences in chromatin.
5
2. The insulator molecule according to claim 1, wherein the insulator is contained within an isolated 1.2 kilobase *SacI-SspI* DNA fragment, or a portion thereof, the DNA fragment comprising the chicken-derived 5' HS4 constitutive hypersensitive site.
10
3. The insulator molecule according to claim 2, wherein the 1.2 kilobase DNA fragment contains a DNA nucleotide sequence according to Seq ID No.:1, or a portion thereof.
15
4. An isolated DNA molecule comprising a chromatin insulator having the sequence according to Seq ID No.:1, or a portion thereof.
20
5. An isolated insulator DNA element; wherein the insulator element is contained within an isolated 1.2 kilobase *SacI-SspI* DNA fragment, or a portion thereof, and the isolated DNA fragment corresponds to the distal 5' constitutive hypersensitive site of the chicken globin locus.
25
6. The insulator molecule according to claim 1, claim 2, claim 3, or claim 4, wherein the *cis*-acting regulatory sequences are enhancers or silencers.
30
7. An expression construct comprising the insulator molecule according to any one of claims 1, 2, 3, or 4.
8. The construct according to claim 7, wherein the construct is operable
35

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when inserted into the DNA of a cell to insulate the expression of one or more genes from one or more *cis*-acting regulatory sequences in chromatin.

9. The construct according to claim 8, wherein the cell is a mammalian cell.

10. A vector construct comprising:

(a) one or more isolated insulator DNA molecules according to any one of claims 1, 2, 3, or 4;

(b) a promoter domain;

(c) a gene operably linked to the promoter domain; and

(d) an enhancer domain 5' of the promoter domain and operable to enhance the activity of the promoter domain and the transcription and expression of the gene; wherein one or more of the insulators is positioned 5' of the enhancer and the promoter domains, and 3' of the gene so as to operably insulate the transcription and expression of the gene from *cis*-acting regulatory elements in chromatin.

11. The construct according to claim 10, wherein two insulators are positioned 5' of the promoter and enhancer and two insulators are positioned 3' of the gene.

12. The construct according to claim 10, wherein the insulator element comprises an isolated 1.2 kilobase *SacI-SspI* DNA fragment corresponding to the distal 5' constitutive hypersensitive site of the chicken globin locus, or a portion thereof.

13. An isolated DNA construct for incorporation into a host cell and for insulation of the expression of a gene therein, comprising:

a) DNA comprising a transcription unit comprising an expressible gene, a promoter to drive the transcription of the gene, and an

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enhancer element; and

b) one or more insulator elements, the insulator element being positioned in sufficient proximity to the transcription unit and to the gene to insulate the transcription and expression of the gene from *cis*-acting DNA regulatory sequences in chromatin.

5

14. The DNA construct according to claim 13, wherein the insulator comprises all or a portion of the insulator element comprising Seq ID No:1.

10 15. The DNA construct according to claim 13, wherein the expressible gene is a structural gene.

15 16. The DNA construct according to claim 13, wherein the expressible gene is a non-structural gene selected from the group consisting of protein-encoding genes, hormone-encoding genes, enzyme-encoding genes, and antibiotic-resistance-encoding genes.

20 17. The DNA construct according to claim 16, wherein the expressible gene is the neomycin-resistance gene or the hygromycin-resistance gene.

25 18. A mammalian cell stably transfected with the construct according to claim 7.

19. A kit for insulating the expression of a transfected and expressed gene, comprising plasmids or vectors comprising the insulator molecule according to any one of claims 1 to 4.

30

20. A pharmaceutical composition comprising the construct according to claim 7 in a pharmaceutically acceptable carrier or excipient.

35

21. A method for insulating the expression of a gene from *cis*-acting

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DNA sequence regulatory elements in chromatin, comprising:

- a) providing the construct according to claim 10; and
- b) transfecting a eukaryotic cell with the construct.

5 22. An isolated DNA construct for insulating the differential expression of two genes, comprising:

- a) one or more isolated eukaryotic insulator molecules according to any one of claims 1 to 4;
- b) a first expressible gene;
- 10 c) a second expressible gene;
- d) a promoter operably linked to the first gene;
- e) a promoter operably linked to the second gene; and
- f) an enhancer operably linked to the second gene;

15 wherein one or more of the insulators is positioned in the construct 3' of the enhancer and 5' of the promoter operably linked to the first gene; wherein the enhancer is positioned 5' of the promoter operably linked to the second gene positioned in opposite transcriptional orientation; and further wherein one or more

20 of the insulators is positioned at the 3' termini of the first and second genes.

23. A DNA construct for insulating differential levels of expression of two genes encoding two different proteins or two genes encoding two distinct subunits of a protein, comprising:

- 25 a) one or more isolated eukaryotic insulator molecules according to any one of claims 1 to 4;
- b) a first expressible gene;
- c) a second expressible gene;
- 30 d) a promoter operably linked to the first gene;
- e) a promoter operably linked to the second gene; and
- f) a first enhancer operable to enhance the expression of the first gene; and
- 35 g) a second enhancer operable to enhance the expression of the

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second gene;

wherein one or more insulators is positioned between the first and the second enhancer; wherein the first enhancer is operable to enhance the activity of the promoter of the first gene in a 5' to 3' direction; and wherein the second enhancer is operable to enhance the activity of the promoter of the second gene in a 5' to 3' direction; and further wherein one or more insulators is positioned at the 3' termini of the first and the second genes.

24. A method for insulating the expression of a transfected gene from a *cis*-acting DNA regulatory sequence in the surrounding chromatin in cells, comprising:

- a) isolating the gene to be transfected and expressed in the transfected cells;
- b) providing a vector construct, comprising:
 - i) the transfectable gene;
 - ii) one or more isolated eukaryotic insulator elements;
 - iii) a promoter operably linked to the gene;
 - iv) an enhancer; wherein one or more insulator elements is positioned 5' of the promoter and the enhancer and 3' of the gene so as to insulate the expression of the gene after integration into cellular DNA; and
- c) transfecting cells with the construct so as to incorporate the construct into the cellular DNA, wherein the transfected insulator element isolates the transcription and expression of the transfected gene from *cis*-acting regulatory sequences in chromatin.

25. A method for preventing a *cis*-acting regulatory element from influencing the transcription and expression of gene in a cell transfected with the gene, comprising:

- a) providing a construct, comprising
 - i) one or more isolated eukaryotic insulator elements;
 - ii) a transfectable gene;

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iii) an enhancer; and
iv) a promoter operably linked to the gene; wherein one or more insulator elements is positioned 5' of the enhancer and the promoter and 3' of the gene so as to insulate the expression of the gene after integration into cellular DNA; and

5 b) transfecting cells with the insulator construct so as to insulate transcription and expression of the transfected gene from *cis*-acting chromatin regulatory elements.

10 26. A method for insulating the expression of a gene from *cis*-acting DNA regulatory sequences in chromatin, comprising:

- a) providing the construct according to claim 11; and
b) transfecting a eukaryotic cell with the construct.

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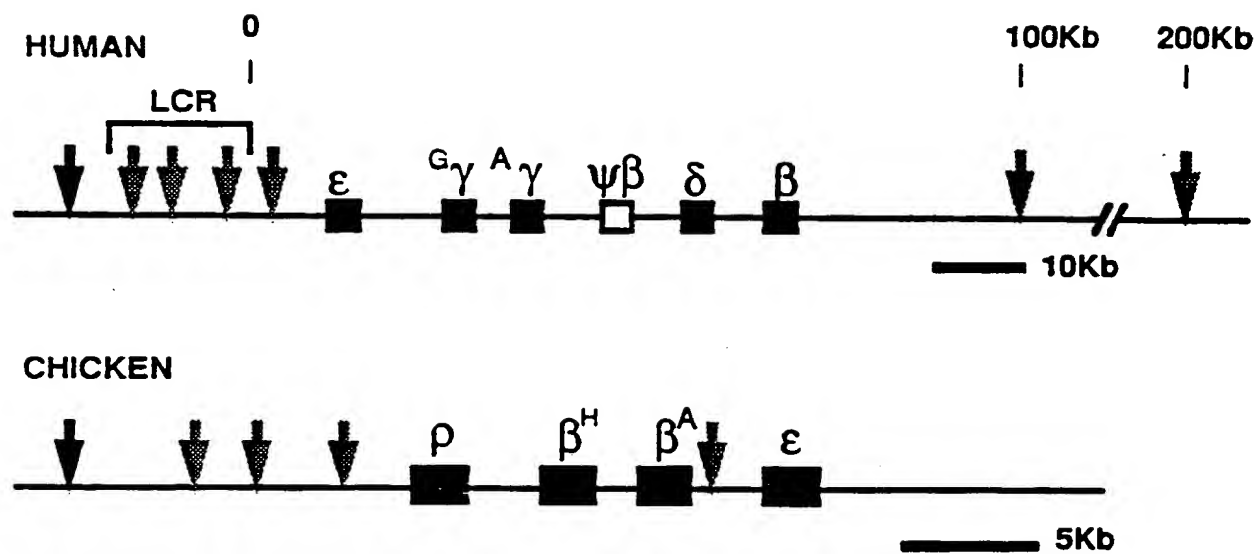


FIG. 1

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AGGGACAGCCCCCCCCAAAGCCCCCAGGGATGTAATTACGTCCCTCCCCCGCTAGGGCA 60

GCAGCGAGCCCGGGGCTCCGGTCCGGTCCGGCGCTCCCCCGCATCCCCGAGCCGGCA 120

GCGTGCGGGGACAGCCCGGGCACGGGGAAGGTGGCACGGGATCGCTTTCCTCTGAACGCT 180

TCTCGCTGCTCTTTGAGCCTGCAGACACCTGGGGGGATACGGGGGAAAAAGCTTTAGGCTGA 242

FIG. 2

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Fig. 3A

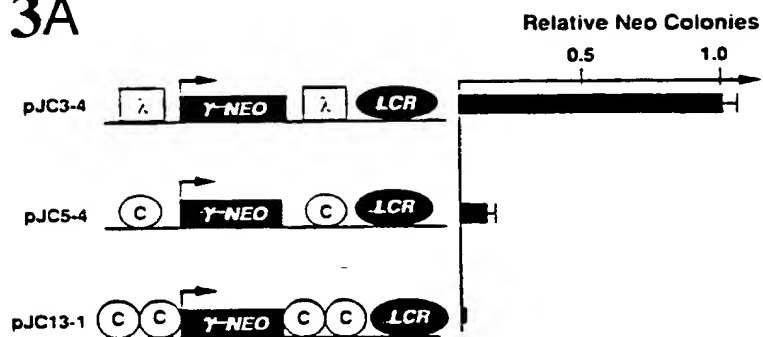
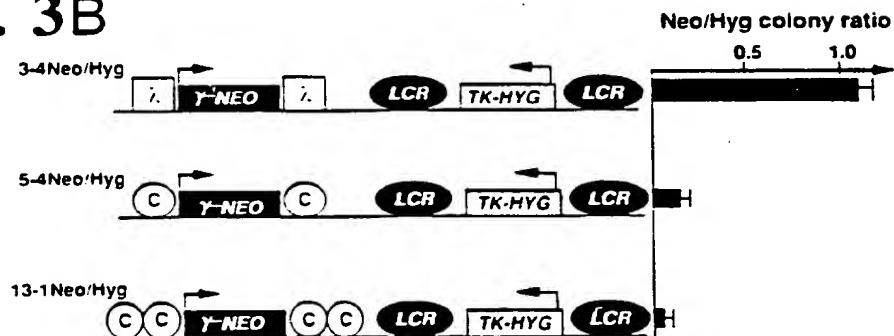


Fig. 3B



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FIG. 4A

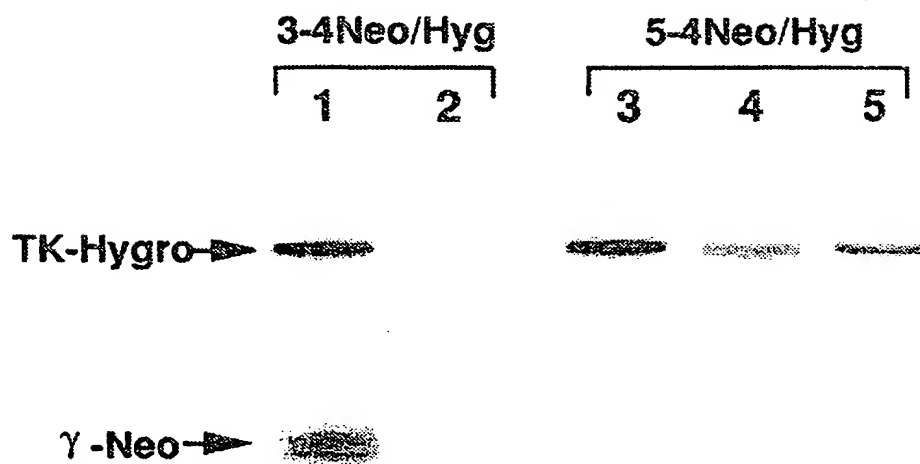
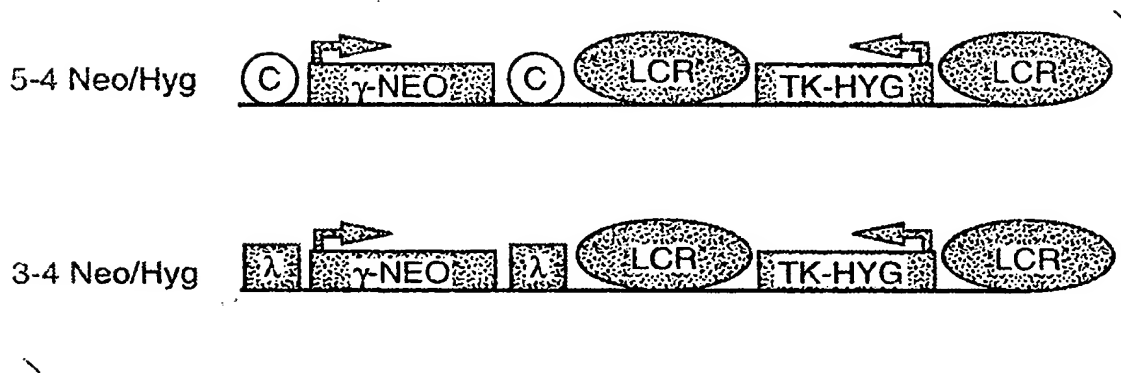
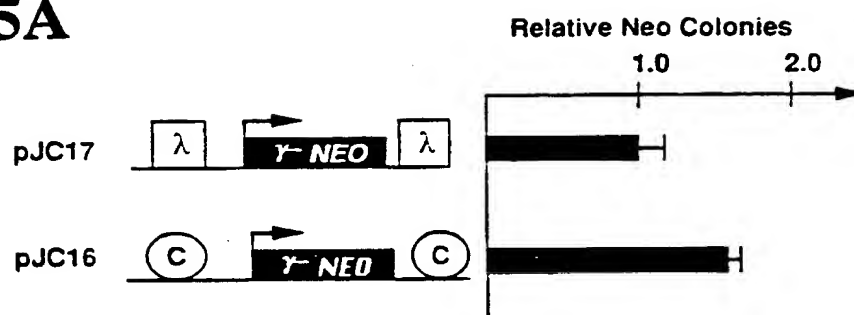
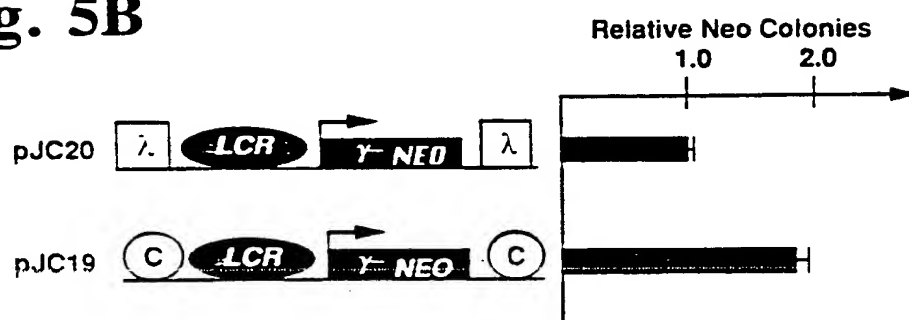


FIG. 4B



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Fig. 5A**Fig. 5B**

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FIG. 6A

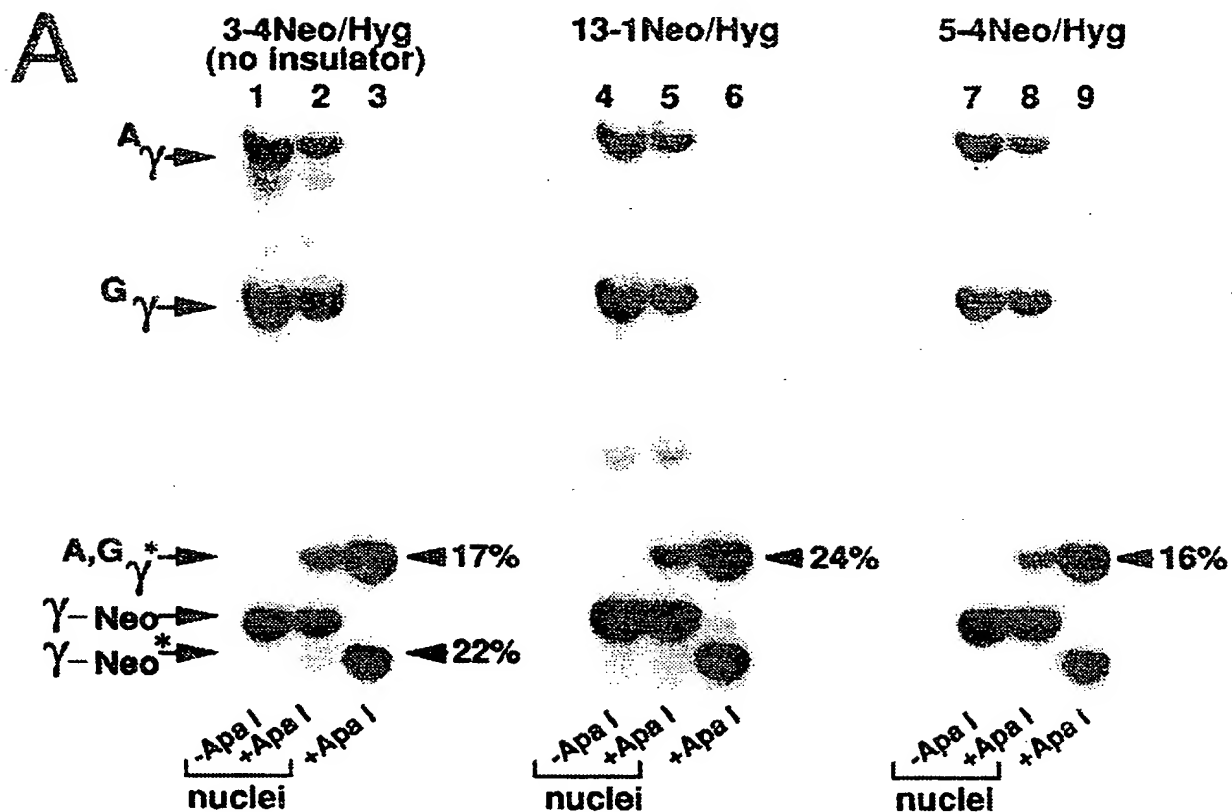
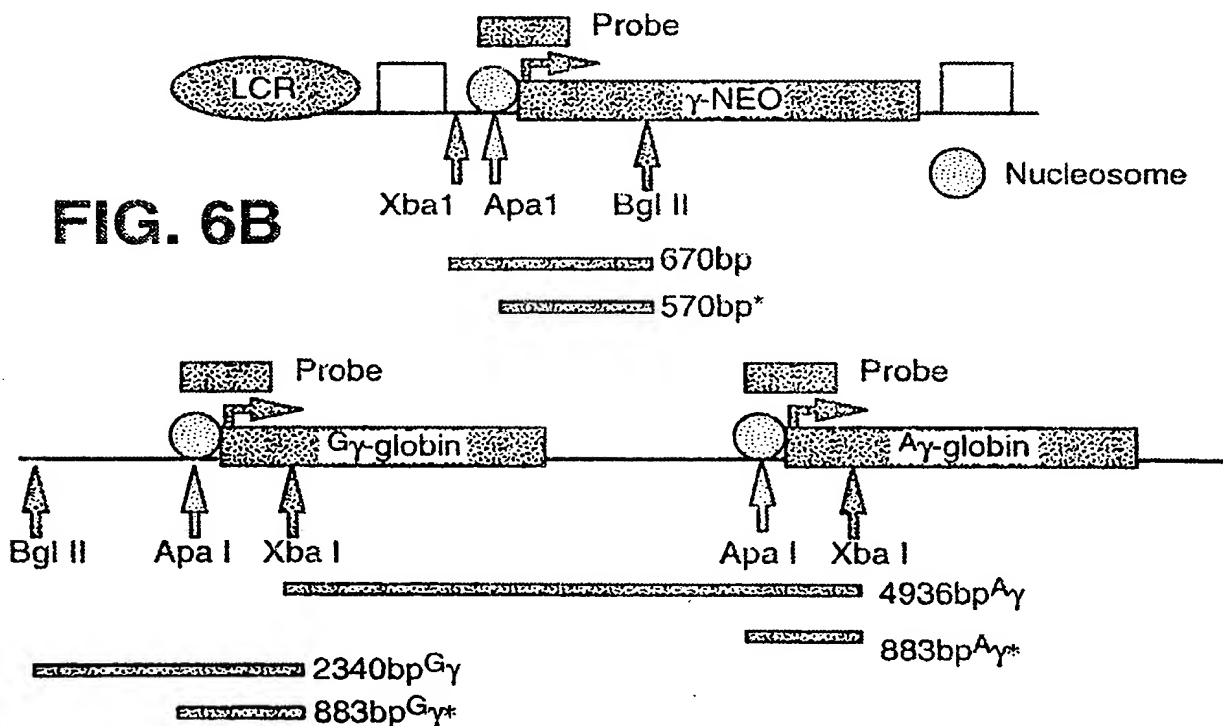


FIG. 6B



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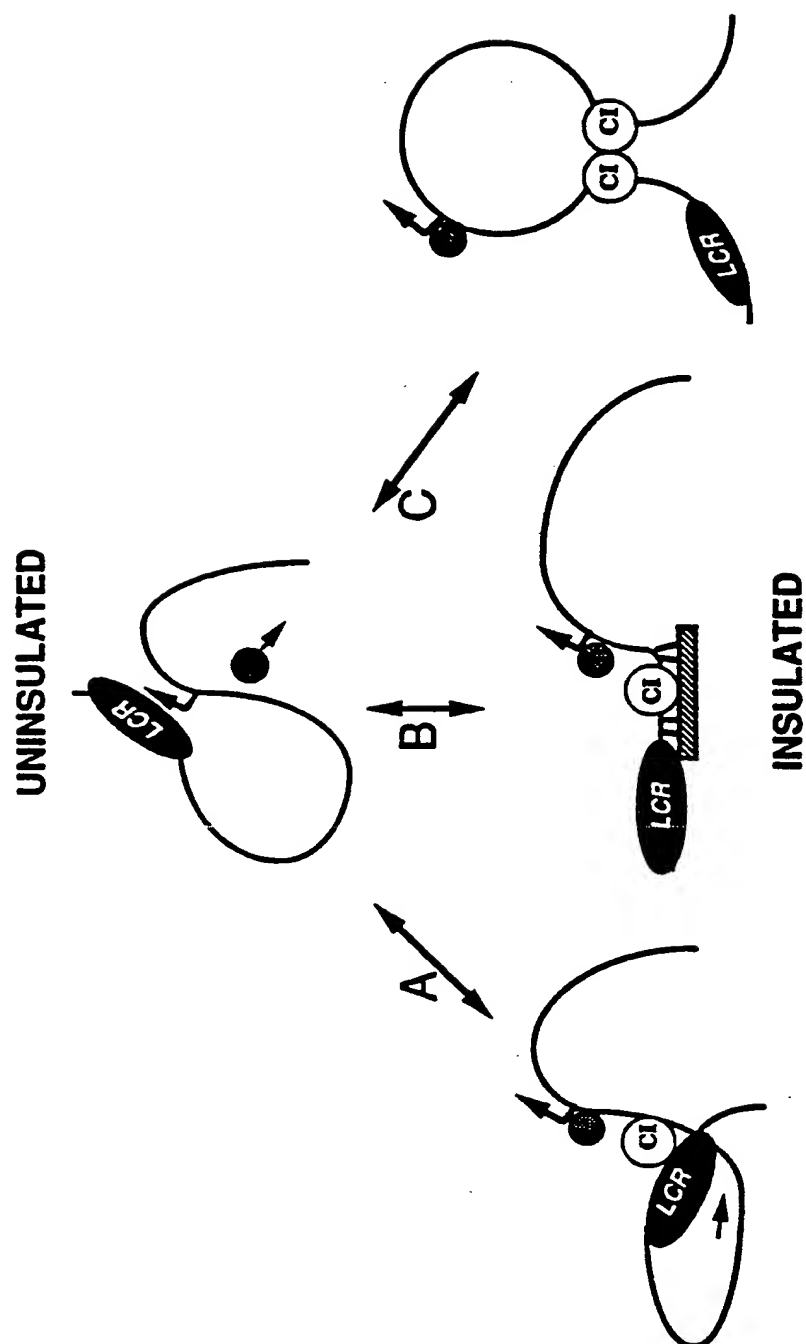


FIG. 7

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/85 C12N5/10 C12Q1/68 A61K31/505

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOLEC. CELL. BIOL. vol. 10 , 1990 pages 2774 - 2786 M. REIEMAN AND G. FELSENFELD; 'Developmental regulation of topoisomerase II sites and DNase I-hypersensitive sites in the chicken beta-globin locus' cited in the application see abstract, Figure 1 and discussion. ---	1-6
X	CELL vol. 64 , 1991 pages 941 - 950 R. KELLUM AND P. SCHEDL 'A position-effect assay for boundaries of higher order chromosomal domains' cited in the application see summary, Figures 1 and 2 and page 945. --- -/--	13,15, 16,24,25

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

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Date of the actual completion of the international search

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Date of mailing of the international search report

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE vol. 341 , 1989 pages 343 - 345 A. STIEF ET AL.; 'A nuclear DNA attachment element mediates elevated and position-independent gene activity' cited in the application see abstract and page 344. ---	13, 15-17, 24,25
A	PROC. NATL. ACAD. SCI. USA vol. 82 , 1985 pages 6384 - 6388 D. TUAN ET AL.; 'The "beta-like-globin" gene domain in human erythroid cells' cited in the application see abstract and pages 6384-5. ---	1
P,X	CELL vol. 74 , 1993 pages 505 - 514 J.H. CHUNG ET AL.; 'A 5' element of the chicken beta-globin domain serves as an insulator in human erythroid cells and protects against position effect in Drosophila' cited in the application see the whole document. -----	1-26

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